Properties of Rat Liver UDP-Glucuronyl Transferase Enzyme Activity

by

Aparna Pandey

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ABSTRACT

Zearalenone (Z) is a hydrophobic xenobiotic which is metabolized by two different reactions, a reduction to an alcohol and a conjugation with glucuronic acid. Experiments were conducted to determine the UDP-glucuronyl transferase (UDPGT) isoenzyme which catalyzes Z conjugation, and the effect of increased enzyme activity on Z metabolism. In competitive enzyme assays, the activity of rat liver UDPGT towards Z was inhibited by 1-naphthol (NA), a GT_1 substrate, and 4-hydroxybiphenyl (HP), a GT_2 substrate. When enzyme activity was induced with either 3-methylcholranthrene (3-MC), a GT_1 inducer, or phenobarbital (PB), a GT_2 inducer, increased UDPGT activity towards Z, NA and HP was observed. UDPGT induction by PB increased urinary excretion of conjugated α-zearalenol. These results indicate that UDPGT isoenzymes have overlapping substrate specificities, and that Z detoxification may be enhanced by UDPGT enzyme induction resulting in increased urinary excretion of conjugated α-zearalenol.

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CONTENTS

Appendix Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Contamination of food and feedstuffs by the secondary metabolites produced by molds is a common problem (Wilson, 1982). Several species of Fusarium molds produce one such metabolite, zearalenone. The temperate Canadian climate is very favorable for zearalenone production (Harwig and Munro, 1975).

Zearalenone is a mycotoxin that naturally occurs in the food chain. It acts as a non-steroidal estrogen like substance having anabolic properties when administered to farm animals in small amounts. However, at high concentrations that can occur in the food and feedstuffs, its effects are toxic (Hidy et al., 1977). Zearalenone has been implicated in numerous incidences of hyperestrogenism; prolonged estrus, anestrus, changes in libido, infertility, increased incidence of psuedopregnancy, increased udder or mammary gland development and abnormal lactation. 'Vulvovaginitis' occurs in the prepubertal gilts (Mirocha et al., 1971), the most sensitive species tested. Kallela and Ettala (1984) have linked zearalenone contaminated hay with

early abortions in the cow. Rats appear to be moderately sensitive to the toxic effects of zearalenone (Kumagai and Shimizu, 1982).

Zearalenone is metabolized by two different reactions, a reduction to an alcohol and a conjugation with glucuronic acid (Kiessling and Petterson, 1978). While conjugation deactivates zearalenone, its reduction increases its potency producing α-zearalenol. Both reduction and conjugation of zearalenone increase its solublity which may promote its excretion in the urine.

Conjugation of zearalenone is catalyzed by the enzyme UDP-glucuronyltransferase (UDPGT) which consists of a family of related isoenzymes (Bock et al., 1982). However, the specific isoenzyme responsible for zearalenone conjugation has not been documented (Olsen et al., 1987).

The objectives of this study were:

(a) To identify the UDPGT enzyme form responsible for zearalenone conjugation;

(b) To increase the UDPGT enzyme activity by induction of enzyme synthesis; and

(c) To observe the effect of induction on the amount of conjugated zearalenone and/or metabolites excreted in the urine.

CHAPTER II

LITERATURE REVIEW

2.1 NUTRITIONAL TOXICOLOGY

Nutritional toxicology is an area of research concerned with toxicants in the diet and their interrelations with nutrition. It is a branch of both toxicology and nutrition concerned with the diet as a source of toxicants, the effects of toxicants on nutrients and nutritional processes, the effects of nutrients and nutritional metabolism on toxicants, and the scientific basis for regulatory decisions affecting toxicological safety of dietary components (Hathcock, 1982). Relationships between nutrition and toxicology fall into three major categories:

(a) the effect of nutritional status on the toxicity of foreign compounds, drugs and environmental chemicals;

(b) the additional nutritional demands that result from exposure to drugs and environmental chemicals; and

(c) the presence of toxic substances in foods (Parke and Ioannides, 1981).

Living beings are exposed to a wide variety of foreign chemicals including natural products, food additives, drugs, insecticides, industrial chemicals and pollutants. All foreign compounds, which gain entrance to the body are collectively termed as xenobiotics. The processes involved in the elimination of xenobiotics are related to those systems which are involved in the elimination of the waste products from the organisms. The biogenic xenobiotics include food poisons such as cyanogens, goitrogens, fish and shell fish poisons, agents causing ergotism and lathyrism and carcinogenic compounds such as aflatoxins.

The main goals of toxicology, and certainly nutritional toxicology, are prevention of toxic action and treatment of eventual intoxication. The hazard that a certain agent presents to man depends on its toxicity (the dose required to produce toxic action) as well as environmental factors such as its distribution, the probablity of contact, the circumstances under which the contact takes place and the physiochemical properties of the agent (Ariens and Simonis, 1982).

The mechanism of toxic actions can be grouped into three categories:

(a) toxic action based on a reversible interaction between the toxicant and the molecular action sites;

(b) toxic effects based on an irreversible, covalent interaction between the toxicant and its target molecules; and

(c) toxicity based on physical sequestration due to accumulation of highly lipid-soluble, metabolically stable compounds in lipid-rich tissues (Hathcock, 1982).

The generation of biological effects of xenobiotic occurs in three distinct phases:

(a) the exposure phase including the physical breakdown of dosage form and solublization of active substance;

(b) the toxicokinetic phase comprised of the absorption, distribution, metabolism and excretion of the xenobiotic; and

(c) the toxicodynamic phase which covers the interaction of the toxicant with its molecular sites of action and the sequence of biochemical and biophysical events thus induced and finally leading to the toxic effect observed (Hathcock, 1982).

2.2 ZEARALENONE

2.2.1 General Characteristics

Molds thrive on organic materials and produce a wide variety of metabolic byproducts. Some of these products man has found a use for, such as enzymes, food flavorings,

solvents and antibodies. However, mold growth on feeds and food stuffs may produce substances that are toxic to animals or humans. These substances are referred to as mycotoxins (Wilson, 1982).

Zearalenone was first isolated from the cultures of Gibberella zeae (Fusarium roseum) by Stob et al., (1962) and was identified as a compound effective in improving growth-rate and feed efficiency in sheep. It is a mycotoxin that naturally occurs in the food chain. It is produced by several species of Fusarium molds endemic to the temperate Canadian climate. Zearalenone belongs to a rare class of natural products, the β-resorcylic acid lactones and is chemically known as 6(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcylic acid lactone.

Zearalenone is a non-steroidal compound whose major biological effects are estrogen-like. It acts as a weak estrogen having anabolic properties when administered to livestock and mice. However, at high concentrations that can occur naturally in foods and feedstuffs, its effects are toxic (Hiddy et al., 1977). The clinical effects of subacute zearalenone mycotoxicosis include decreased feed intake, reduced growth and decreased reproduction (Mirocha et al., 1980). Zearalenone causes an estrus syndrome called vulvovaginitis involving inflammation of the vulva and the posterior part of the vagina, when fed to the swine (Mirocha et al., 1971). It caused retarded growth and reduced carcass

weight in both male and female rats (Kiessling, 1982). Kallela and Ettala (1984) have linked early abortions in cows in Finland to the hay feed contaminated with zearalenone. Shoental (1983) suggested that zearalenone may be a factor in human precocious sexual development in Puerto Rico. Limited dietary exposure of female swine to zearalenone can result in embryonic loss and disruption of normal reproductive cycling for an extended length of time (Long et al., 1983).

Zearalenone and its metabolites can accumulate in the adipose tissue, liver, uterus and other organs of rats (Ueno et al., 1977).

2.2.2 Biological Activities

Zearalenone and its metabolites, despite their phenolic chemical structure, possess a chemical similarity to estradiol. Estradiol and these mycotoxins have similar chemical solubility and close spatial similarities (Hurd, 1977; Lindsay, 1985). Alpha-zearalenol and estradiol are both 10-11A^{O} in length and are of similar lipophilicity. Similarly, the substance Mentzer's coumarin has a chemical structure and anabolic activity homologous to that of α-zearalanol, the commericial anabolic zearalenone derivative (Hurd, 1977). These molecules are approximately the same length, differing by only 1-2A^{O}, and both compounds

possess similar oxygen containing functional groups at the end of their ring systems. The distance between the pair of oxygen functions for both the substances is about 11A^{O}. Thus, the estrogenicity of zearalenone and other compounds may be a result of the placement of atoms relative to one another within the molecule, in a manner similar to estrogen (Hurd, 1977).

Zearalenone and its metabolites are capable of binding to and interacting with the estrogen receptors (Lindsay, 1985). The ability of these compounds to bind to the uterine cytosolic estrogen receptors is a measure of their relative potencies. Alpha-zearalenol is the most potent stimulator of uterine growth, followed by zearalenone and β-zearalenol (Ueno and Tashiro, 1980). This can be correlated with the greater binding affinity of α-zearalenol towards the estrogen receptors (Picken et al., 1989).

The interspecies differences observed in the sensitivity to zearalenone have been explained by the differences in the relative binding affinities of this compound for the estrogen receptors in the pig, rat and the chicken (Picken et al., 1989). Pigs are most sensitive to zearalenone because their estrogen receptors have the greatest affinity for these mycotoxins. Even a single exposure to the toxin at concentrations as low as 5 mg kg^{-1}body weight has been found to produce visible signs of toxicosis (Farnworth and Trenholm, 1981). Rats are moderately sensitive to effects

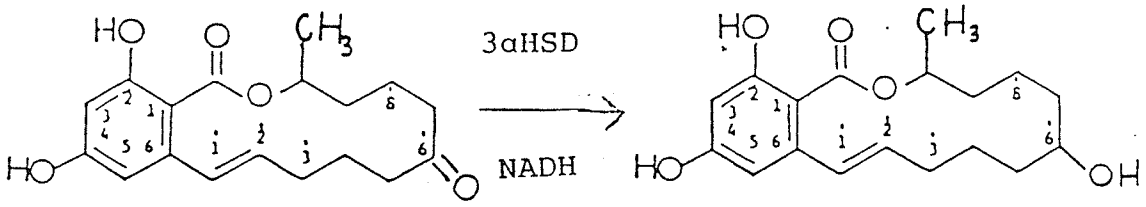
of zearalenone, while poultry are relatively refractory. Administration of a 1 mg dose of zearalenone to neonatal rats caused anovulatory estrus (Kumagai and Shimizu, 1982). Dietary zearalenone, as high as 800 mg kg^{-1} body weight, fed to chicken and turkeys had no effect on body weight gain, feed consumption and organ weight (Allen et al., 1981).

2.2.3 Metabolism

Kiessling and Petterson (1978), demonstrated that zearalenone was metabolized along two principal pathways, a reduction to produce zearalenol and/or a conjugation with glucuronic acid. The metabolism of zearalenone is a classic example of Phase I and Phase II reactions. In the Phase I reaction, the enzyme 3α-hydroxysteroid dehydrogenase (E.C.1.1.1.50) catalyzes the reduction of zearalenone to either α- or β-zearalenol. Picken et al., (1989) demonstrated that the relative binding affinity of α-zearalenol was ten fold greater than zearalenone, which suggests that it was more toxic than zearalenone, and that this reduction is an activation reaction. Zearalenone can also be reduced to β-zearalenol. Picken et al., (1989) suggested that this was a deactivation reaction, since the relative binding affinity of β-zearalenol for estrogen

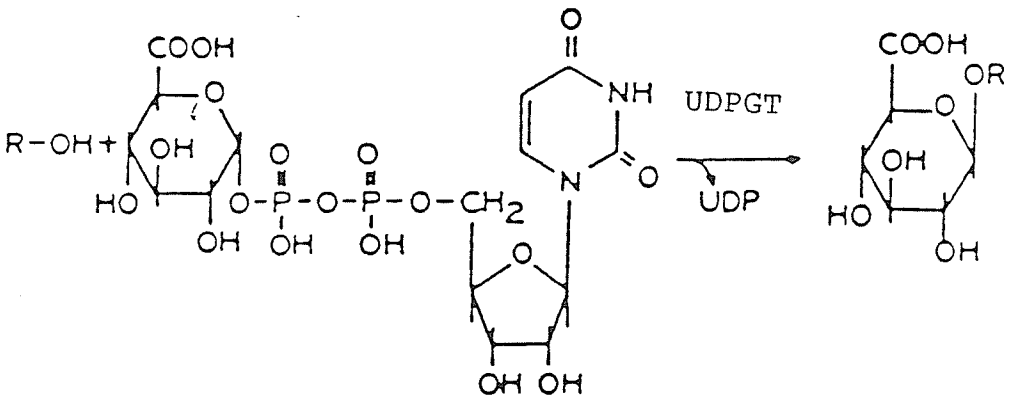
ZEARALENONE IS METABOLIZED BY TWO DIFFERENT ROUTES:

1. Reduction of zearalenone to zearalenol,
   (Phase I reaction)



Zearalenone Zearalenol

2. Conjugation with UDP-glucuronic acid,
   (Phase II reaction)



(Kiessling and Petterson, 1978).

Figure 2.1 Metabolism of zearalenone

receptors was ten fold less than the parent compound. Thus the Phase I reaction of reduction can lead to either biological activation or deactivation.

In the Phase II reaction, conjugation with glucuronic acid takes place and the reaction is catalyzed by the enzyme UDP glucuronyl transferase. It was demonstrated that the addition of UDP-glucuronic acid increased the amount of conjugated zearalenone in a preparation of rat liver homogenate and zearalenone. This conjugation was prevented when β-glucuronidase, an enzyme responsible for the breakdown of conjugates, was added in the incubation medium. This proved that the enzyme UDP glucuronyl transferase (UDPGT; E.C.2.4.1.17) was responsible for the conjugation of zearalenone (Kiessling and Petterson, 1978).

Conjugation deactivates zearalenone. Here it can be noted that zearalenols can also be conjugated to some extent (Kiessling and Pettersson, 1978). Recent studies by Kiristy et al., (1987), and Fitzpatrick et al., (1988a) suggest that nutritional regimen effects zearalenone metabolism, excretion and toxicity. That is by increasing the total amount of zearalenone and its metabolites excreted as glucuronide conjugates in the urine, the toxic expression of zearalenone would be ameliorated.

Quantitatively the reduction of zearalenone has been thought to be a less important metabolic route when compared

to conjugation. In an *in vitro* study with rat liver homogenate, the amount of conjugated zearalenone and its metabolites was observed to be two to five times more than the reduced zearalenone (Kiessling and Petterson, 1978). However, Fitzpatrick et al., (1988a) reported the activities of 3α-hydroxysteroid dehydrogenase and UDPGT to be 150 and 50 nanomoles $hour^{-1}mg^{-1}$protein. This observation suggests that the reduction pathway is the more important metabolic route.

Olsen et al., (1985) have demonstrated in pigs that zearalenone and its metabolites are present in the plasma and are excreted in the urine mainly in a bound form as glucuronide conjugates. In the plasma, the concentrations of α-zearalenol were three to four times higher than that of the parent compound when dietary zearalenone was administered to prepubertal gilt. Given the biopotency of α-zearalenol, and the conflicting evidence on the importance of the two metabolic routes, the question of metabolic excretion rates warrants additional investigation.

2.2.4 Enterohepatic circulation

Once zearalenone enters the liver it may be reduced to α-zearalenol or conjugated with UDP-glucuronic acid. Zearalenone and its metabolites can move out of the hepatic veins into the general circulation and exert their effects

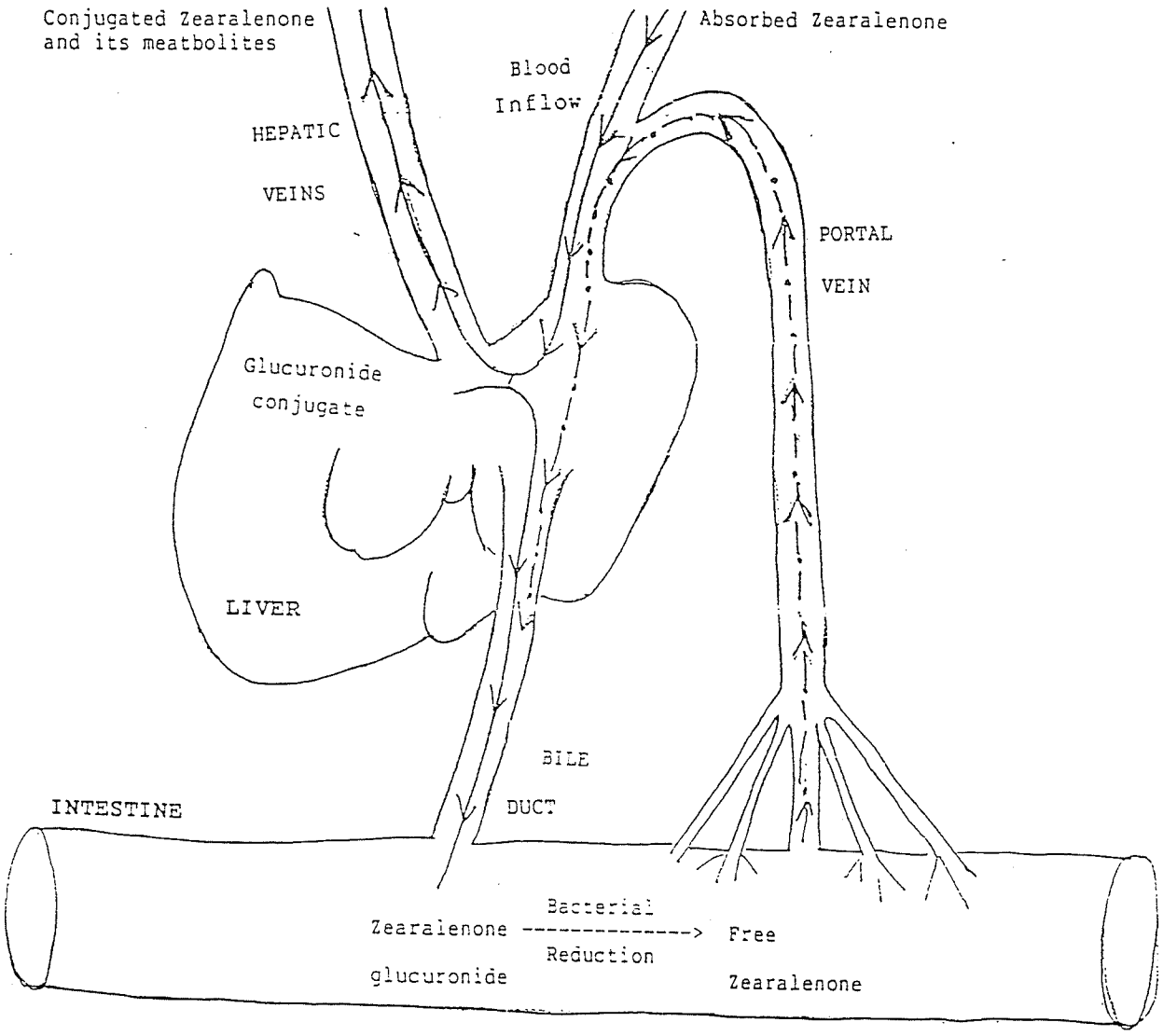


Figure 2.2 Zearalenone excretion and enterohepatic recycling

on the target tissues. Conjugation of zearalenone or its metabolites by the addition of glucuronic acid, deactivates the compound. From the liver the deactivated metabolites can either be directed to kidney via hepatic veins and excreted in the urine or they can be transported to the intestine via the bile duct. Conjugated metabolites may be broken down to free zearalenone or zearalenol by the enzyme β-glucuronidase produced by the intestinal microflora. Free zearalenone/metabolites can be excreted in the feces or reabsorbed and transported to the liver via portal circulation. This cycle, from liver to intestine via bile duct and back to liver via portal vein, is called enterohepatic recycling. Enterohepatic recycling increases the biological half life of zearalenone. Conjugation and urinary excretion limit enterohepatic circulation of zearalenone, reduce its biological half life, and may act to limit the toxic expression of zearalenone (Kiritsy et al., 1987, Fitzpatrick et al., 1988a).

2.2.5 Excretion of zearalenone

Zearalenone and its metabolites are excreted in urine and feces, mainly in the free form with conjugates being observed only in the urine (Kiritsy et al., 1987 and Smith, 1982). Conflicting reports concerning the route of zearalenone excretion and the major metabolites formed have

been published (Ueno, 1977; Smith, 1980; Smith, 1982; Kiritsy et al., 1987; Fitzpatrick et al., 1988a). Table 2.1 summarizes studies on the zearalenone excretory patterns reported in the rat.

Fitzpatrick and coworkers (1988a) observed significant quantities of zearalenone and its metabolites, 25-51% of the oral dose, were excreted in the urine. This was in agreement with previous studies from their laboratory (Kiritsy et al., 1987), but were in contrast to the latest findings of Smith (1982). Fitzpatrick and coworkers suggested that this apparent discrepancy was due to differences in the dose level and recoveries, and that the use of pharmacological doses in toxicity experiments could result in erroneous conclusions. They concluded that the overdosing of animals results in `metabolic overload' with the ability of the body to detoxify the chemical exceeded. The rate and/or route of excretion may be significantly altered. For example, Smith (1982) dosed his animals with 10mg zearalenone per 100g body weight and recovered 3% of the total dose in the urine and 22% in the feces for a total recovery of 25% of the oral dose. Fitzpatrick et al., (1988a) dosed rats with 1mg zearalenone per kg body weight and recovered 64% of the oral dose, 26% in the urine and 38% in the feces.

However, dose level alone may not account for the reported discrepancies. Smith (1980) dosed rats with 10mg

Table 2.1 Studies on zearalenone excretion in the rats.

Author	Dose ($mg\ Kg^{-1}$ bw)	Total Recovery (% Adm. dose)		
		Urinary	Fecal	Total
Fitzpatrick et al.,1988a	1	26	38	64
Kiritsy et al.,1987	1	33	32	65
Smith 1982	100	3	22	25
Smith 1980	100	31	62	93

per 100g body weight of [^{3}H]-zearalenone and recovered 31% of the radioactivity in the urine and 62% in the feces. Recently, Fitzpatrick et al., (1988b) reported that dose level had little effect on zearalenone metabolism, and suggested that the discrepancies in the literature may be due to differences in recoveries.

Zearalenone and its metabolites are sparingly soluble in water, which is not conducive to their urinary excretion (Hidy et al., 1977). Conjugation of zearalenone with glucuronic acid increases its molecular weight and also increases its solublity in aqueous medium (Caldwell, 1982). The molecular weight threshold of urinary excretion of water soluble material is 325 $\pm$ 50. Below this threshold compounds are excreted in the urine, while materials with molecular weights above this threshold are excreted in the bile. Zearalenone, with a molecular weight of 318, is near the threshold and can be excreted in either the urine or the bile. Conjugation increases the molecular weight and this may cause an increase in the biliary excretion. However, the water solubility is increased as well, which may result in an increased urinary excretion.

2.3 GLUCURONIDATION IN DETOXIFICATION

2.3.1 Introduction

Many foreign compounds entering the body undergo metabolic transformations. These transformations may play an important role in any therapeutic action or toxicity such compounds exert. The metabolism of xenobiotics is a biphasic process. Initially the compound undergoes Phase I, or functionalization reaction of oxidation, reduction or hydrolysis, in which a polar group such as -OH, $-NH_2$ or -COOH is attached to the molecule. Phase I reactions may lead to detoxification, but can also produce toxic intermediates extremely damaging to the organism. The products of Phase I reactions are substrates for Phase II, where functional group from Phase I facilitate conjugation. Phase II reactions are biosynthetic in nature. The xenobiotic is linked with an endogenous moiety to give a characteristic product known as `conjugate' (Williams, 1959). In certain cases, the xenobiotic possess a functional group suitable for Phase II metabolism and will directly undergo conjugation reactions (Williams, 1967). The reactions proceeds as follows:

Functionalization Conjugation

Compounds ----------> Phase I ----------> Phase II

metabolite metabolite

reaction reaction

(Caldwell, 1985)

The Phase II reactions differ from Phase I reactions in:

(a) the nature of the enzymes involved in the two phases;

(b) Phase II reactions are all synthetic reactions involving expenditure of energy; and

(c) the products of Phase I reactions are still partially lipid soluble and may posses biological activity, while the products of Phase II are usually biologically inactive.

The decreased activity of the products of Phase II can be attributed to increased water solublity allowing its rapid removal from the body and a masking of biologically active groups by superimposition of, or stereochemical hindrance by the conjugating molecule (Dutton, 1980).

The Phase II reactions require energy and an endogenous molecule. For example, for glucuronidation, the organism

must supply ATP, a source of uridine to be phosphorylated and a source of carbohydrate. The reactions proceed as follows:

ATP + UDP ----------> UTP + ADP

ATP + Glucose ----------> Glucose-6-P + ADP

or ATP + Glucose ----------> Glycogen + Glucose-1-P

UTP + Glucose-1-P ----------> UDP-Glucose + Pyrophosophate

UDP-Glucose + NAD^+ ----------> UDP-glucuronic acid + NADH

UDP-glucuronic acid + ROH --------> R.O.glucuronide + UDP

(Dutton, 1980)

The major conjugation reactions of xenobiotic metabolism are glucuronidation, sulphation, methylation, acetylation, cyanide detoxification and glutathione conjugation. Glucuronidation is the most wide spread form of conjugation in all vertabrate species and accounts for most of the conjugated detoxicatory material in bile and urine (Smith and Williams, 1966). There are two main reasons that explain the widespread utilization of glucuronidation in vertebrates. Glucuronic acid is widely available substrate, in that it is derived fairly directly from the universal vertebrate fuel, glucose. Glucuronic acid can be conjugated with a remarkably wide range of molecular groupings (Dutton, 1980).

Glucuronidation causes an increase in polarity of the resultant metabolite, which makes it more soluble in bile or urine. Once in blood or bile, they will remain in the aqueous environment and will not readily pass through the lipid membranes, into the cells. From blood they will be filtered by kidney and pass down the tubules without reabsorption by the tubule cells to appear almost quantitatively in the urine (Dutton, 1980). The bilirubin glucuronidation is a common example of conjugation. Bilirubin is toxic to an infant mammal. It is conjugated with the polar molecule glucuronic acid and is rendered water soluble, thus it is available for biliary excretion (Dutton, 1980).

2.3.2 Excretion of glucuronides

The majority of the drugs and other foreign compounds are ultimately cleared from the body by their excretion in the urine and bile, usually in the form of polar conjugates such as glucuronides. The glucuronide excretion may occur in one of the three ways either in the urine only or in the bile only or in both urine and bile (Dutton, 1966).

In the rat glucuronides of relatively low molecular weight such as benzoyl glucuronide and p-aminophenol glucuronide are excreted almost exclusively in urine. By contrast, certain glucuronides, particularly those of

relatively high molecular weight are excreted almost entirely in the bile. For example the bilirubin glucuronide is excreted in the bile. The N-glucuronide of sulfadimethoxine is excreted to about equal amounts in both the urine and the bile. In some cases one of the two routes may predominate as in the case of the excretion of alpha-tetralyl and vanilloyl glucuronides. These two conjugates are excreted in both the urine and bile of rats but elimination in the urine is the major route of excretion (Dutton, 1966).

2.3.3 Influence of diet on glucuronidation

Dietary restriction affects UDPGT and glucuronidation. Caloric restriction may reduce the availablity of UDP-glucuronic acid, while protein restriction changes the membrane composition and hence the activity of UDPGT enzyme (Grahm et al., 1974). Marselos and Laitinen (1974) observed that stimulation of transferase activity by phenobarbital pretreatment was greater in starved animals, starved for three days. Kiritsy et al., (1987) reported that feed restriction resulted in a doubling of both the UDPGT enzyme activity, and the amount of conjugated urinary metabolites.

2.4 THE ENZYME UDPGT

2.4.1 General Reaction

The enzyme uridine diphosphate glucuronyl transferase (UDPGT) (EC 2.4.1.17), catalyzes the translocation of glucuronic acid from UDP-α-D glucuronic acid to an appropriate acceptor, to form the β-D-glucuronide. Inversion occurs at the anomeric carbon atom of glucuronic acid to form the β-D-glucuronide. The R-group that attaches to the glucuronic acid, is hydrophobic in nature, and is either aliphatic or aromatic. Introduction to the glucuronyl residue increases the polarity of the molecule and also contributes a carboxyl group, which exists primarily in the unprotonated form at pH of the most physiological fluids. This allows salt formation and facilitates excretion in either bile or urine (Kasper and Henton, 1980).

2.4.2 Location

UDPGT has been observed in all mammals, birds and reptiles, although the spectrum of acceptor specificities for various species differ widely (Dutton, 1980). In mammals liver exhibits the greatest total amount of UDPGT enzyme activity as well as the highest specific activity. Other tissues that are active in glucuronidation are kidney,

gastro-intestinal tract and skin. These tissues share the impact from the animal's environment (Dutton, 1966; Kasper and Henton, 1980). At the cellular level, UDPGT was first observed in the cytoplasmic granules of liver homogenates (Dutton and Storey, 1954). Subsequent studies have shown that UDPGT exists as a lipid-protein complex, firmly integrated into the phospholipid bilayer of the endoplasmic reticulum (Kasper and Henton, 1980). Gram et al., (1968) observed the non-uniform distribution of transferase activities in the microsomal subfractions. Most of the activity towards simple phenols was in the rough microsomes. Activities towards phenolphthalein, bilirubin and steroids have been reported in smooth microsomal fractions (Wishart et al., 1978). The UDPGT enzyme activities have been reported in liver, kidney, small intestinal mucosa, lung, skin, tests and spleen (Bock et al., 1980).

2.4.3 Induction

It is generally accepted that toxicities from many compounds including certain drugs, pesticides, hepatoxins and chemical carcinogens can be altered by induction or inhibition of certain hepatic microsomal enzymes (Hodgson and Guthrie, 1980). It has been suggested that induction is enhanced enzyme activity after administration of various agents. It is an adaptation to the xenobiotic which

increases the total cellular content of a certain protein (Bock et al., 1979; Astrom et al., 1987). While there is constant protein turnover in a cell there can be increased synthesis based upon specific structures. Induction is, therefore, a slow process.

The protein which acts to increase the observed level of enzyme activity may be the enzyme itself or another protein, which controls expression of the enzyme activity. The protein can exert its control in the following ways:

(a) by slowing the enzyme breakdown;

(b) by facilitating the access of one or more substrates of the enzyme;

(c) by releasing an activator; or

(d) by removing an inhibitor.

Thus induction reflects a change in concentration of an enzyme protein or of environmental proteins determining its activity (Dutton and Burchell, 1975).

Two inducers for the enzyme UDPGT have been reported as 3-methylchloranthrene and phenobarbital (Induction Studies, 2.5.2).

2.4.4 Activity

In freshly prepared homogenates, the activity of enzyme UDPGT is latent. Activation of fresh enzyme occurs on storage (Burchell et al., 1975). This enzyme is activated by

membrane pertubations such as mechanical disruption, aging, freezing, thawing or exposure to detergents, chaotropes, organic solvents, alkali, certain ions, proteases or phospholipases (Dutton 1980). Berry et al., (1975) have proposed that mechanical procedures like sonication, blending and grinding with glass, activated the enzyme by simply rupturing the vesicles and improving substrate accessibility.

2.4.5 Definition of Activation

Activation is different from induction in that it is an increase in the enzyme activity, under conditions precluding protein synthesis. It directly reflects change in the activity of preformed protein molecules whose catalytic potency as UDPGT is partially or wholly unexpressed (Dutton and Burchell, 1974).

Here activation has been explained as :

(a) removal of the barriers that restrict the access of the reactants into the catalytic site which is embedded in the hydrophobic membrane; and

(b) Conformational change in the enzyme protein which makes it easier for the substrate to bind to the catalytic site (Dutton, 1980).

2.4.6 Storage

At $0^{o}C$, liver homogenates lose UDPGT activity after one hour (Dutton, 1966). In contrast, activity to p-nitrophenol in rat liver microsomes, increased eight folds, when stored for eight days at $0^{o}C$. Storage of an activated transferase resulted in a progressive decline in enzyme activity (Lucier et al., 1971). Activation on storage could result from endogeneous bile salt, liberation of peroxides, lysolecithin and free fatty acids and/or disruption of the membranes by proteases or phospholipases. It is markedly temperature dependent (Burchell et al., 1975).

2.4.7 Breakdown of Glucuronides

The enzyme β-glucuronidase (EC 3.2.1.21) catalyzes the hydrolysis of the biosynthetic β-glucuronides to yield their various aglycones and free glucuronic acid. Under all normal circumstances, β-glucuronidase liberates the aglycone and glucuronic acid in equivalent amounts and the equilibrium point lies far in the favor of hydrolysis (Wakabayashi and Fishman, 1961). Beta-glucuronidase activity is often inversly proportional to the glucuronyl transferase activity in tissues, physiological states and drug treatment (Mulder, 1971; Lucier and McDaniel, 1972). The enzyme is used for the identification of conjugate as a β-glucuronide of the substrate (Dutton et al., 1981).

Glucaro(1-4)lactone is a powerful inhibitor of β-glucuronidase activity in liver and kidney and is employed to establish the presence of β-glucuronidase in impure preparations. The lactone is considered as a potential drug in diseases in which it is suspected that there may be a disorder of glucuronic acid metabolism (Dutton, 1980).

2.5 CLASSIFICATION OF UDPGT

2.5.1 Introduction

Recently, researchers have recognized that glucuronidation is catalyzed by a family of related isoenzymes (Bock et al., 1983; Chowdhury et al., 1985). They suggest that :

(a) the UDPGT isoenzymes have similar molecular weight and are usually separable on the basis of charge, but their primary protein structures are very different from each other.

(b) there is overlapping substrate specificity with in the family of the isoenzymes.

(c) the halflife of the isoenzymes is similar, approximately twenty four hours.

(d) the enzyme activity is regulated by increased synthesis of the enzyme.

Classification of the various isoenzymes of UDPGT has been based on the following criteria :

(a) response to different inducers;

(b) rates of development;

(c) different rates of enzyme reaction as indicated by K_m and V_{max}; and

(d) physical separation.

2.5.2 Induction Studies

Bock et al., (1973) suggested that several forms of UDPGT exist in rat liver microsomes. They demonstrated that phenobarbital (PB) increased the glucuronidation of bilirubin and chloramphenicol whereas 3-methylcholanthrene (3-MC) stimulated the glucuronidation of phenolic substrates such as p-nitrophenol and 1-naphthol. Similar observations on the heterogeneity of the enzyme were made by Wishart (1978b) with a wider variety of substrates.

Bock et al., (1980) introduced a nomenclature for the two purified enzyme forms of UDPGT that are differentially inducible by 3-MC and PB and named them GT_1 and GT_2 respectively. The GT_1 activity was detectable in liver, kidney, small intestinal mucosa, lung, skin, testes, and spleen, whereas the GT_2 activity was only present in liver

TABLE 2.2 Classification of UDP-glucuronyl transferase activities based on Induction studies.

	GT_1	GT_2	References
SUBSTRATES	p-nitrophenol 1-naphthol	bilirubin chloramphenicol	Bock et al., (1973)
INDUCERS	3-MC	PB	
SUBSTRATES	1-naphthol p-nitrophenol 4-methylumbe- -lliferone 5-OHtryptamine 2-aminobenzoate	bilirubin chloramphenicol morphine testosterone phenolphthalein estradiol	Wishart (1978b)
INDUCERS	3-MC	PB	
SUBSTRATES	1-naphthol N-OH 2-naphth- -yl amine 3-OHbenzo(a)- -pyrene	4-OHbiphenyl chloramphenicol morphine	Bock et al., (1980)
INDUCERS	3-MC	PB	
SUBSTRATES	1-naphthol 4-methylumbe- -lliferone	4-OHbiphenyl morphine	Lilienblum et al., (1982)
INDUCERS	β-naphthoflavone	DDT	
SUBSTRATES	1-naphthol furosemide	estrone furosemide	Rachmel and Hazelton (1986)
INDUCERS	3-MC -	PB PCN	

continued

(Table 2.2 continued)

	GT_1	GT_2	References
SUBSTRATES	1-naphthol benzo(a)pyre- -ne quinol	4-OHbiphenyl morphine bilirubin	Koster et al., (1986)
INDUCERS	3-MC	PB	
SUBSTRATES	phenol 4-methylphenol 4-ehtylphenol	4-n-propylphenol 4-s-butylphenol 4-t-butylphenol	Wishart and Campbell (1979)
INDUCERS	3-MC dexamethasone	- -	
SUBSTRATES	1-naphthol p-nitrophenol phenol	4-OHbiphenyl morphine chloramphenicol	Okulicz- Kozaryn et al., (1981)
INDUCERS	3-MC	PB	

and intestine. Similar to 3-MC, another inducer, β-naphthoflavone selectively stimulated the glucuronidation of GT_1 substrates. Dichlorodiphenyl trichloroethane (DDT), on the other hand, enhanced the glucuronidation of GT_2 substrates, similar to PB (Lilienblum et al., 1982).

Rachmel and Hazelton (1986) have attempted to characterize the UDPGT activity towards furosemide in rat liver by inducing the enzyme activity with 3-MC, PB and pregnenolone-16α-carbonitrile (PCN). All inducers, increased furosemide UDPGT activity. These experiments provided evidence that several UDPGT forms are involved in the glucuronidation of furosemide. However, in the same study, the induction of glucuronidation of NA by 3-MC and that of estrone by PB and PCN was also observed. That is, specific inducers increased UDPGT activity towards specific substrates. The study of Koster et al., (1986), on the distribution and induction of UDPGT activities in different organs of the rat, supported the model of differential induction. GT_1 activities for the substrates 1-naphthol, and benzo(a)pyrene-3,6 quinol were induced by 3-MC in liver. The enzyme activities toward morphine and HP were induced by PB in the liver and the intestine. PB induced the UDPGT activities towards bilirubin also. This was detected in the hepatic, intestinal and renal microsomes.

Bock and Lilienblum (1985) combined the measurement of UDPGT activity towards aglycones belonging to different

substrate groups with Immunoblot analysis using rabbit anti-rat liver GT_1 antibodies. They observed an increase in 54 and 56 KDa polypeptides, the major 3-MC inducible UDPGT forms in rat liver which they correlated with a biochemically measurable increase of glucuronidation capacity or induction of UDPGT activity for the GT substrates in hepatic, intestinal and renal microsomes (Koster et al., 1986).

The molecular models of UDPGT substrates studied by Wishart (1978b) indicated that the group of substrates affected by PB induction were longer and more bulky than the small planar substrates induced by 3-MC (Wishart and Campbell, 1979). To pursue this observation further, these workers investigated transferase activities towards a series of 4-alkyl-substituted phenols, ranging from simple planar molecules of phenol to the more bulky 4-t-butylphenol. Two groups of substrates were distinguished on the basis of induction with 3-MC and dexamethasone. They observed that even a difference of a single $-CH_2$ moiety was sufficient to change the acceptability of these substrates from one group of transferase activity to another. Thus, the two groups of transferase activities do not distinguish substrates on the basis of their molecular weights or lipophilicity but due to the differences in the specific molecular configuration of the substrates.

Okulicz-Kozaryn et al., (1981) have extended the idea of 'specific molecular configurations' first proposed by Wishart and Campbell (1979). They observed, that the thickness or the bulkiness of the molecules play an important role in the classification of substrates based on induction of enzyme activity by 3-MC or PB. The molecules more affected by 3-MC had their estimated thickness less than 4 A^{O}, while for the PB-induced UDPGT group, molecular thickness was greater than 4 A^{O}.

Boutin et al., (1984) demonstrated a correlation between the conjugation velocity of the enzyme and the chemical nature of the substrates. A strong conjugation activity was observed towards p-nitrophenol, 4-methylumbelliferone, 1-naphthol, p-bromephenol and eugenol, all planar molecules. The conjugating activities in the second group of substrates were 5-7 times weaker. This group of substrates included 4-hydroxybiphenyl, morphine and chloramphenicol, all bulky molecules. It can be noted here that the first group of substrates correlates with the GT_1 group while the second group correlates with the GT_2 group of substrates (Bock et al., 1980).

2.5.3 Isolation and Purification Studies

Several research groups have established the

heterogeneity of the UDPGT enzyme by physically separating the various enzyme activities. Del Villar et al., (1975) using DEAE-cellulose chromatography, separated morphine and p-nitrophenol UDPGT isoenzymes from rat liver microsomes. Three peaks of activity were eluted, with one isoenzyme displaying activity with p-nitrophenol, another showed the activity with morphine, and the third displayed activity with both substrates. These results were confirmed by Gorski and Kasper (1977) who purified liver microsomal UDPGT using anion exchange chromatography and affinity chromatography with UDP-hexanolamine Sepharose 4B. Three forms of the enzymes were resolved by isoelectric focussing.

Bock et al., (1979), using DEAE cellulose chromatography and affinity chromatography on UDP-hexanolamine sepharose 4B, separated and purified two isoenzymes of UDPGT. After pretreatment with the inducing agents, it was observed that the isoenzyme induced by 3-MC was active towards 1-naphthol, p-nitrophenol, 3-hdroxyl-benzo(a)pyrene and N-hydroxy-2-naphthylamine. While, the PB-induced enzyme form showed activity towards morphine chloramphenicol and 4-hydroxybiphenyl.

Matern et al., (1982) observed that hepatic microsomal UDPGTs with activity toward chenodeoxycholic acid and testosterone were homogeneous in sodium dodecyl sulfate gel electrophoresis and polyacrylamide gradient gel electrophoresis. This indicated that chenodeoxycholic acid

and testosterone were glucuronidated by a single form of the enzyme. UDGPT activities towards estrone, bilirubin, p-nitrophenol and morphine did not co-purify with the activity toward chenodeoxycholic acid and testosterone, suggesting that they were conjugated by a different isoenzyme.

Falany and Tephly (1983) purified three UDPGT isoenzymes using chromatofocussing, column isoelectric focussing and UDP-hexanolamine sepharose 4B affinity chromatography. In this study, two separate isoenzymes capable of conjugating p-nitrophenol were detected. One of these isoenzymes was not inducible by 3-MC and was also active towards testosterone and 17-OH position of β-estradiol. A third isoenzyme conjugated androsterone and etiocholanole and was not induced by 3-MC treatment.

Falany et al.(1986) further characterized these three isoenzymes. The two steroid UDPGT isoenzymes were renamed 17β-hydroxysteroid and 3α-hydroxysteroid UDPGT to reflect their specificity for important endogenous substrates. No endogenous substrate could be identified for the third, p-nitrophenol specific (3-MC inducible), UDPGT isoenzyme. The peptide maps generated by limited proteolysis with Staphylococcus aureus V_8 proteinase showed that the three purified UDPGTs were separate and distinct proteins.

In a purification study (Chowdhury et al., 1985), six enzymically active peaks were eluted after chromatography of

the microsomal fraction of rat livers at different pHs. The peak I (pH 8.9) isoenzyme of UDPGT had no endogenous substrate of its own. The peaks II (pH 8.4) and III (pH 8.0) isoenzymes had activity for the 17-OH steroids, testesterone and β-estradiol. The peak IV (pH 7.8) isoenzyme had activity for the 3-OH androgenic steroid, androsterone. The peak VI was active towards the 3-OH steroid estrone and β-estradiol. Also estradiol was glucuronidated in the 17-OH position by the peak II isoenzyme and in the 3-OH position by the peak VI isoenzyme. The peak V (ph 7.5) was specific for bilirubin glucuronidation.

Puig and Tephly (1986) used trisacryl DEAE and chromatofocussing chromatographic procedures to purify morphine specific UDPGT enzyme activity. The purified enzyme reacted with morphine but not with 4-hydroxybiphenyl, p-nitrophenol, testosterone, androsterone, estrone, bilirubin, 4-aminobiphenyl or α-naphthylamine.

2.5.4 Developmental Studies

An investigation of the heterogeneity of UDPGT was carried out by Wishart (1978a), who determined the enzyme activity in the rat liver during the perinatal period. The enzyme activities towards the substrates, 2-aminophenol, 2-aminobenzoate, p-nitrophenol, 1-naphthol, 4-methylumbelliferone and 5-hydroxytryptamine increase to

TABLE 2.3 Classification of UDP-glucuronyl transferase activities based on developmental studies.

Late Fetal group	Neonatal group	References
1-naphthol	morphine	Wishart
4-nitrophenol	chloramphenicol	(1978a)
4-methylumbelliferone	bilirubin	
2-aminophenol	testosterone	
2-aminobenzoate	β-estradiol	
5-hydroxytryptamine	phenolphthalein	
phenol	4-n-propylphenol	Wishart and
4-methylphenol	4-s-butylphenol	Campbell
4-ethylphenol	4-t-butylphenol	(1979)

reach adult values between the days 16 to 20 of gestation. Thus this group of substrates was named the late foetal group. The activities towards bilirubin, testosterone, β-estradiol, morphine, phenolphthalien and chloramphenicol remain negligible at less than 10% of adult values, during this late foetal period. The enzyme activity towards this group of substrates, termed the neonatal group, approached adult values by the second postnatal day. It was suggested that foetal glucocorticoids were responsible for triggering the increase in the hepatic UDPGT activities in the late foetal group. They suggested that, the neonatal group of activiites required an additional stimulus for their appearance.

Wishart (1978b) demonstrated that the late foetal group of substrates were conjugated by the UDPGT isoenzyme that was inducible by 3-MC. And that the group of substrates with their enzyme activities induced by PB correlated with the neonatal group of substrates in the developmental study.

Wishart and Campbell (1979) distinguished two groups of transferase activities towards a different set of substrates, on the basis of their onset of development. Activities towards phenol, 4-methylphenol and 4-ethylphenol develop near-adult values before birth and are assigned to a 'late-foetal' group of transferase activities. Activities towards 4-n-propylphenol, 4-s-butylphenol and 4-t-butylphenol are negligible in late-foetal liver and

develop to near-adult values in the first four post natal days. These are assigned to the neonatal group of transferase activities. It was observed that the former transferase activity is stimulated by 3-MC and dexamethasone while the latter activity was not affected by either of the two inducers.

2.5.5 Kinetic Studies

Analysis of UDPGT affinity towards different aglycones and towards UDP-glucuronic acid has provided evidence of different molecular forms of UDPGT, which have different substrate specificities. In a study of kinetic properties of UDPGTs in rat liver cells, Antoine et al., (1984) observed that glucuronidation of 4-nitrophenol was characterized by high V_{max} and high affinity for UDP-glucuronic acid, while conjugation of morphine exhibited a low V_{max} but the enzyme had high affinities for both UDP-glucuronic acid and the aglycone. The conjugation of borneol had a low V_{max} as well as a low affinity for UDP-glucuronic acid.

Antoine et al., (1985) next studied the localization of the UDPGT enzyme along with its kinetic properties. They suggested that more than one UDPGT may exist in the different membranes which have different catalytic properties. The GT_1 form of UDPGT enzyme conjugated planar molecules and was preferentially located in the endoplasmic

reticulum where it presented the maximum glucuronidation capacity (high V_{max} and high affinity for UDP-glucuronic acid). The second GT form GT_2, was involved in the conjugation of the bulkiest molecules, it had a low V_{max} and was equally distributed in all the subcellular locations.

Thus, there have been two forms of UDPGT isoenzymes, that are clearly identified, characterized and their tissue distribution reported, but there may be many more forms of UDPGT. The isoenzyme GT_1 is specific for planar substrates for example 1-naphthol and 4-nitrophenol and is preferentially inducible by 3-methylcholanthrene. The isoenzyme activity has been detected in the liver, kidney, small intestinal mucosa, lung, skin, testes and spleen and it increases to reach adult values between the days 16 and 20 of gestation. The second isoenzyme GT_2 is specific for bulky substrates for example morphine and 4-hydroxybiphenyl and is preferentially inducible by phenobarbital. This isoenzyme activity has been detected in the liver and intestine. It reaches adult values by the second postnatal day.

Chapter III

MATERIALS AND METHODS

3.1 ANIMALS

Female Sprague-Dawley rats (90-110g) were purchased from the University of Manitoba central breeding facility. Animals were housed separately in stainless steel cages and kept on a 14-10 hour light-dark cycle. Animals were fed a commercial chow diet (Ralston Purina Co., St.Louis, USA). Animals were randomly assigned to the various experimental groups.

3.2 EXPERIMENTAL PROTOCOL

3.2.1 Substrate Stimulation Study

3.2.1.1 Animals

Each day, ten animals were humanely killed and their liver homogenate prepared. The homogenate was pooled together and its protein concentration determined using the method described by Lowry (1951). This was assayed for

UDPGT, each assay being performed in duplicate. The UDPGT activity was expressed as nM acceptor substrate conjugated per h per mg protein.

3.2.1.2 UDPGT Enzyme Assay : Spectrophotometric Analysis

The method was as described by Mulder and Van Doorn (1975) (Appendix A). Six sets of substrates were tested in this assay, namely:

(1) 1-naphthol (GT_1-specific substrate)

(2) 4-hydroxybiphenyl (GT_2-specific substrate)

(3) Zearalenone

(4) 1-naphthol and zearalenone

(5) 4-hydroxybiphenyl and zearalenone

(6) 1-naphthol and 4-hydroxybiphenyl.

3.2.1.3 UDPGT Enzyme Assay : HPLC Analysis

The assay was an adaptation (Appendix B) of that reported by Mulder and Van Doorn (1975). The sets of substrates tested were the same as described in 3.2.1.2.

3.2.2 Induction Study

3.2.2.1 Animals

Ten animals were used for each group, control or experimental. The animals received intra-peritoneal doses of

the inducers or the vehicles for three days. On the fifth day, they were humanely killed, liver excised, and immediately assayed for UDPGT.

3.2.2.2 Inducers

The rats received an i.p. treatment with PB, 3-MC or the appropriate control vehicle as follows. PB: An initial dose of 30 mg Kg^{-1}body weight in saline, was given on day 1, and 60 mg Kg^{-1}body weight on day 2 and 3. The controls received 0.89% saline on all three days. The two compounds were administered to the rats in a total volume of 2 ml Kg^{-1}body weight. 3-MC: An i.p. dose of 30 mg Kg^{-1}body weight in corn oil was given and the controls received corn oil only. Both of these compounds were administered in a total volume of 3 ml Kg^{-1}body weight.

3.2.2.3 Enzyme Assays

These were as described in sections 3.2.1.2 and 3.2.1.3. The substrates tested in this assay were,

(1) 1-naphthol

(2) 4-hydroxybiphenyl

(3) Zearalenone.

3.2.3 Metabolic Study

3.2.3.1 Animals

The number of animals used were twelve and eleven for the control and the experimental groups, respectively. After receiving i.p. doses of phenobarbital or its vehicle, saline for three days, the animals were dosed with 5 mg kg^{-1} body weight of zearalenone by stomach intubation on the fifth day. Animals were transfered to polycarbonate metabolic cages (Nalgene, Fisher Scientific), urine was collected every 24 hours for four days and was kept frozen at $-20^{o}C$ until analyzed. Zearalenone and its metabolites were extracted and analyzed by HPLC (Fitzpatrick et al., 1988b).

3.2.3.2 Inducer

Phenobarbital produced a similar increase in UDPGT activity as observed in the results from the induction study (Table 4.3 and 4.4) as did diet induction (Fitzpatrick et al., 1988a). The other inducer used in the induction study, 3-methylcholanthrene is known to be a potent cytochrome P_{450} inducer. Therefore phenobarbital was selected as an inducer for this study.

The inducer was administered using the same protocol as described in 3.2.2.2.

3.2.3.2 HPLC Methods

The method used in this study was as developed by Fitzpatrick et al., (1988b) (Appendix C).

3.3 STATISTICAL ANALYSES

Data was analyzed using the Statistical Analysis System (SAS), 1982 version. For the Substrate Stimulation study, means were calculated on the treatment groups and compared using a paired t-test. For the Induction and the Metabolic studies, significant differences were estimated by Student's t-test (control versus experimental).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 SUBSTRATE STIMALATION STUDY

The enzyme UDPGT constitutes a family of related isoenzymes, and of a number of UDPGT isoenzymes proposed, two have been unambiguosly characterized. These two enzyme forms, GT_1 and GT_2 glucuronidated specific substrates (Bock et al., 1980). NA has been reported as being a specific GT_1 substrate, while HPis thought to be a GT_2 substrate. The specific isoenzyme responsible for zearalenone conjugating activity is not yet identified. The objective of this study was to identify the zearalenone conjugating isoenzyme of UDPGT.

The active site of an enzyme represents a small part of the enzyme molecule. At saturating substrate concentrations the amount of enzyme and thereby the availablity of active sites will be rate limiting. Substrates compete for the active sites on the enzyme, therefore when two or more substrates are present in an

enzyme preparation, the rate of metabolic transformation is reduced by substrate competition. This is known as Competitive Inhibition.

In the present work, zearalenone was considered the substrate for the reaction, while NA and HP, substrates specific for the two different GT forms were considered as potential inhibitors. Presence of a certain substrate in the assay medium with zearalenone will inhibit the enzyme activity towards zearalenone if both zearalenone and that substrate are conjugated by the same enzyme form. On the other hand, no inhibition should occur if the substrates are conjugated by different UDPGT isoenzymes.

We have modified the indirect assay of Mulder and Van Doorn (1975) to examine substrate competition. The Substrate Stimulation study done by this assay was based on the principle that :

(1) If two substrates x and y are conjugated by different UDPGT isoenzyme forms then, the sum of the enzyme activities towards the substrates x and y will be equal to the enzyme activity when the two substrates are together in the medium.

$$\{ x \} + \{ y \} = \{ x + y \}$$

There is no competition for active sites on the enzyme and therefore the enzyme activities are additive.

(2) However, if two substrates x and y are conjugated by the same UDPGT isoenzyme form then the sum of the enzyme activities towards the substrates x and y will be more than the enzyme activity when the two substrates are together in the medium.

$$\{ x \} + \{ y \} > \{ x + y \}$$

There is competition for active sites because both the substrates are conjugated by the same isoenzyme form and the two enzyme activities are not additive.

In the indirect assay, the enzyme acitivities for zearalenone, NA and HP were 109, 282 and 108 nmol NAD^+ formed $h^{-1}mg^{-1}$protein respectively. The observed activity when both zearalenone and NA were present in the reaction medium was 263 nmol NAD^+ formed $h^{-1}mg^{-1}$protein. This was significantly different from the theoretical sum of enzyme activities for these two substrates (391 nmol NAD^+ formed $h^{-1}mg^{-1}$protein) ($p<0.01$) (Table 4.1). This indicates that the UDPGT enzyme activity towards zearalenone is affected by the presence of NA, suggesting that the two substrates are conjugated by the same isoenzyme form of UDPGT. In the direct analysis of individual substrates by HPLC, conjugation of zearalenone was 251 and 181 nmol glucuronide formed $h^{-1}mg^{-1}$protein when alone and with NA, respectively, in the reaction medium ($p<0.05$) (Table 4.2).

TABLE 4.1 Substrate Stimulation Study. Effect of Z, NA, HP and combinations of these acceptor substrates on UDP-glucuronyl transferase enzyme activity.

	UDP-glucuronyl transferase activity	
Substrate(s)	Observed	Calculated+
Z	109 ± 20	
NA	282 ± 41	
HP	108 ± 16	
Z and NA	263 ± 43	391 ± 61*
Z and HP	98 ± 24	217 ± 36*
NA and HP	272 ± 76	390 ± 58*

Each value is expressed as nmol NAD^+ formed per h per mg protein and represents the mean ± SD of 3 pooled assays.

+ Calculated values are the theoretical sum of UDPGT enzyme activity towards the individual acceptor substrates.

* Significantly different from observed value ($p<0.01$).

Z: zearalenone,
NA: 1-naphthol,
HP: 4-hydroxybiphenyl.

TABLE 4.2 Substrate Stimulation Study. Glucuronidation of Z, NA, HP and combinations of these acceptor substrates substrates by UDP-glucuronyl transferase as determined directly by HPLC analysis.

	Acceptor substrate glucuronidation		
Substrate(s)	Z	NA	HP
Z	251 ± 8		
NA		ND	
HP			244 ± 37
Z and HP	160 ± 5*		183 ± 25*
Z and NA	181 ± 15*	ND	
HP and NA		ND	217 ± 42*

Each value is expressed as nmol of acceptor glucuronidation per h per mg protein and represents the mean ± SD of 2 pooled assays.

ND, reaction too rapid to be determined under assay conditions.

* Significantly different from glucuronidation rate when present in the reaction medium as the only acceptor substrate ($p<0.05$).

Significant differences were observed between the theoretical sum of enzyme activities for zearalenone and HP (109 + 108 = 217 nmol NAD^+ formed $h^{-1}mg^{-1}$protein) and the enzyme activity when the two were in the medium (98 nmol NAD^+ formed $h^{-1}mg^{-1}$protein). ($p < 0.05$) (Table 4.1). The HPLC analysis of enzyme activity towards zearalenone (251 nmol glucuronide formed $h^{-1}mg^{-1}$protein) was significantly more than the enzyme activity towards zearalenone (160 nmol glucuronide formed $h^{-1}mg^{-1}$protein) when HP was also present in the medium.($p < 0.05$). Also, the enzyme activity towards HP alone in the medium (244 nmol glucuronide formed $h^{-1}mg^{-1}$protein) was significantly more than the enzyme activity (183 nmol glucuronide formed $h^{-1}mg^{-1}$protein) when zearalenone was also present in the medium ($p< 0.05$) (Table 4.2). These results demonstrate that zearalenone and HP inhibit each others conjugation and suggest that they are conjugated by the same isoenzyme form of UDPGT.

In order to further test the classification of UDPGT isoenzymes, the substrates NA and HP were incubated together. These substrates have been reported to be specific substrates for two different forms of UDPGT isoenzymes (Bock et al., 1980). The results from the indirect assay demonstrated that the theoretical sum of enzyme activities with NA and HP (282 + 108 = 390 nmol NAD^+ formed $h^{-1}mg^{-1}$protein) was significantly more

when both substrates were simultaneously present in the medium (272 nmol NAD^+ formed $h^{-1}mg^{-1}$protein) ($p<0.01$).

On HPLC analysis, the enzyme activity towards the substrate HP (244 nmol glucuronide formed $h^{-1}mg^{-1}$ protein) was significantly more than the enzyme activity towards HP (217 nmol glucuronide formed $h^{-1}mg^{-1}$protein) when NA was also present in the medium ($p<0.05$). These results indicate that there is competition for active sites on the enzyme between the two substrates allegedly conjugated by two different isoenzymes.

Thus this study does not support the reports that NA and HP are conjugated by two distinctly different isoenzymes of UDPGT, rather a small amount of substrate overlapping is indicated.

Olsen et al., (1987) have found no correlation between the rates of glucuronide conjugation of zearalenone and NA in the sow intestinal mucosa and have therefore suggested that the two substrates may depend upon two different isoenzymes of UDPGT. There is no other evidence of the characterization of UDPGT enzyme form responsible for zearalenone conjugation in the literature.

4.2 INDUCTION STUDY

Glucuronidation reactions have been reported to be differentially inducible by two protypes of inducing agents, 3-Methylcholanthrene (3-MC) and Phenobarbital (PB). Again, NA, reported as a typical GT_1 substrate is preferentially induced by 3-MC administration, while HP, which is thought to be a GT_2 substrate, is preferentially induced by PB (Bock et al., 1980).

In the induction study, 3-MC administration resulted in a significant increase in the UDPGT enzyme activity towards all three substrates. The enzyme activity towards NA increased by 290% from 302 to 1181 nmol of NAD^+ formed $h^{-1}mg^{-1}$protein in the 3-MC induced animals ($p<0.05$) (Table 4.3). The conjugation of zearalenone was increased by 53% from 118 to 181 nmol of NAD^+ formed $h^{-1}mg^{-1}$protein in the 3-MC induced animals ($p<0.05$) (Table 4.3). There was an increase in the enzyme activity towards HP, by 43% from 117 to 167 nmol NAD^+ formed $h^{-1}mg^{-1}$protein in the induced animals ($p<0.05$). This suggests that while 3-MC preferentially induced NA glucuronidation, UDPGT activity towards zearalenone and HP are enhanced as well.

Zearalenone conjugating activity, measured by HPLC analysis, was increased 100%, from 211 to 423 nmol

Table 4.3 Enzyme induction. Effect of phenobarbital or 3-methylchloranthrene pretreatment on UDP-glucuronyl transferase enzyme activity towards Z, NA and HP.

	UDPGT enzyme activity+		
Substrate	Control	3-MC pretreatment	Percentage Stimulation
Z	118 ± 13	181 ± 20*	53
NA	302 ± 65	1181 ± 107*	290
HP	117 ± 19	167 ± 19*	43

	UDPGT enzyme activity+		
Substrate	Control	PB pretreatment	Percentage Stimulation
Z	99 ± 14	138 ± 30*	40
NA	283 ± 73	401 ± 87*	41
HP	109 ± 14	166 ± 31*	52

+ Each value is expressed in nmol NAD+ formed per h per mg protein and represents the mean ± SD of 10 animals.

* Significantly different from controls ($p<0.05$).

TABLE 4.4 Enzyme induction. Effect of phenobarbital or 3-methylchloranthrene pretreatment on glucuronidation of Z and HP as determined directly by HPLC analysis.+

	Substrate glucuronidation		
Substrate	Control	3-MC pretreatment	Percentage Stimulation
Z	211 ± 49	423 ± 13*	100
HP	301 ± 16	396 ± 52*	31

	Substrate glucuronidation		
Substrate	Control	PB pretreatment	Percentage Stimulation
Z	341 ± 205	286 ± 80	0
HP	284 ± 83	441 ± 18*	55

Each value is expressed in nmol of acceptor glucuronidation per h per mg protein and represents the mean ± SD of 3 animals.

+ Glucuronidation reaction with NA as the acceptor substrate was too rapid to be determined under the assay conditions.

* Significantly different from the control value ($p<0.05$).

glucuronide formed $h^{-1}mg^{-1}$protein, due to 3-MC pretreatment ($p<0.05$) (Table 4.4). The enzyme activity towards HP was increased 31%, from 301 to 396 nmol glucuronide formed $h^{-1}mg^{-1}$protein which is comparable to 43% increase detected in the Mulder and Van Doorn assay ($p<0.05$). It is to be noted here that the enzyme activity towards NA could not be measured under experimental conditions used in this study.

Administration of PB, also resulted in an increase in the UDPGT enzyme activity towards all the three substrates. The conjugation of HP increased 52%, from 109 to 166 nmol NAD^+ formed $h^{-1}mg^{-1}$protein in the PB induced animals ($p<0.05$). The enzyme activity towards zearalenone increased 40%, from 99 to 138 nmol NAD^+ formed $h^{-1}mg^{-1}$protein in the induced animals ($p<0.05$). The conjugation of NA increased 41%, from 283 to 401 nmol NAD^+ formed $h^{-1}mg^{-1}$protein in the PB induced animals. In the direct measurement, the enzyme activity towards HP increased 55%, from 284 to 441 nmol glucuronide formed $h^{-1}mg^{-1}$protein, in the PB induced animals ($p<0.05$) (Table 4.4), which is comparable to 52% increase observed in the indirect analysis.

Thus the pretreatment of female rats with both the inducers stimulated hepatic UDPGT activity towards zearalenone.

Our results corresponding to the induction of enzyme form glucuronidating NA generally agree with the literature. Bock et al., (1979) observed that the increase in glucuronidation of NA after administration of 3-MC (40mg/kg) was 182% while the increase due to PB administration was only 12% over their respective controls. Glucuronidation of another substrate, HP increased by 27% after 3-MC treatment and 342% after PB administration (Table 4.5). The UDPGT activities towards the two substrate groups were separated and purified from the livers of rats pretreated with these inducing agents.

A similar classification for UDPGT enzyme activities towards substrates was reported by Okulicz-Kozaryn et al., (1981). They observed an increase in NA-UDPGT activity, after 3-MC (15mg/kg) administration, of 130%, while PB (80mg/kg for three days) increased the enzyme activity 10%. The enzyme activity towards HP was increased by 10% after 3-MC administration and 150% after PB treatment, over their controls (Table 4.5).

The study by Koster et al., (1986) supports the model of differential induction based on selective induction of enzyme activities in rat liver microsomes by 3-MC or PB. The inducer 3-MC (40mg/kg) increased the glucuronidation of NA by 330% and that of HP by 50%. The increase in the enzyme activities by administration of

TABLE 4.5 Comparison of induction values with the studies reported in the literature.

Study	Treatment	Percentage Stimulation
1-Naphthol (GT_1)		
Bock et al., 79	MC	182
(male rats,n=4)	PB	12
Okulicz et al., 81	MC	130
(male rats,n=5)	PB	10
Koster et al., 86	MC	330
(male rats,n=2)	PB	50
Astrom et al., 87	MC	467
(male rats,n=3)	PB	-
(female rats,n=3)	MC	258
	PB	-
Rachmel & Hazelton 86	MC	240
(male rats, n=5)	PB	60
Present Work	MC	290
(female rats,n=10)	PB	41
4-Hydroxybiphenyl (GT_2)		
Bock et al., 79	MC	27
(male rats,n=4)	PB	342
Ockulicz et al., 81	MC	10
(male rats,n=4)	PB	150
Koster et al., 86	MC	50
(male rats,n=2)	PB	230
Present Work	MC	43
female rats,n=10)	PB	52

PB (100 mg/kg followed by 0.1% (w/v) in drinking water) towards NA was only 50% while that towards HP was 230% over their respective controls (Table 4.5). The activity towards GT_1 substrate, NA was detected in the liver, kidney and intestine, while the activity towards GT_2 substrate HP was restricted to the liver and intestine.

Rachmel and Hazelton (1986) studied the characterization of UDPGT activity towards furosemide, a diuretic agent, in rat liver and compared these features with the glucuronidation of NA and estrone. Furosemide glucuronidation was increased by 282%, 208% and 342% of controls by the administration of 3-MC (20mg/kg for four days), PB (75 mg/kg for four days) and pregnenolone-16α-carbonitrile (PCN) (75 mg/kg for four days) respectively. In comparison, NA glucuronidation was preferentially induced by 3-MC (240% stimulation over the controls) while estrone glucuronidation was induced by PB and PCN (390% and 150% stimulation over the controls, respectively). This study suggested that more than one form of UDPGT with different inducibilities is involved in the glucuronidation of furosemide.

Zearalenone fits in this classification of two groups of aglycone substrates, as being glucuronidated by both GT_1 and GT_2 enzyme forms. Overlapping substrate specificity within the UDPGT family of isoenzymes has been suggested by Chowdhury et al., (1985). Conjugation

of furosemide is another example of substrate overlapping between two GT forms one of which is induced by 3-MC and the other being induced by PB as well as PCN (Rachmel and Hazelton, 1986).

The extent of induction of NA glucuronidating enzyme by 3-MC and PB from our results is comparable with the results reported in the literature. However, the increase in the enzyme activity towards HP by the administration of PB was relatively low.

A low enzyme activity towards HP in tissues other than liver and intestine was observed by Bock et al., (1980). The authors have suggested that some overlapping substrate specificity of GT_1 might exist towards HP.

Enzyme form GT_1 appears to selectively conjugate planar compounds whereas enzyme form GT_2 conjugates bulky molecules (Bock et al., 1979). HP is considered a GT_2 substrate since the phenyl group in the para-position favors a non-planar configuration. An equilibrium between planar and non-planar confirmation has been suggested for the molecule (Bock et al., 1980).

The heterogeneity of UDPGT can also be attributed to the thickness of the molecules in their most likely conformation (Okulicz-Kozaryn et al., 1981). These authors have suggested that the estimated thickness of the molecules in their most likely conforamtion was below 4 A^{o} for the GT_1 substrates and more than 4 A^{o}

for the GT_2 substrates. The molecular thickness of NA, a GT_1 substrate has been reported to be 1.7 A^o while for HP, a GT_2 substrate it is 4.3 A^o. According to these values, as well as the bulkiness criteria suggested by Bock et al., (1980), HP is expected to fall on the borderline between GT_1 and GT_2 group of substrates to some extent. Thus some overlapping activity with GT_1 is expected.

The rats used in all the studies discussed were male rats. The effect of inducer treatment on the enzyme activity in male rats may not be representative for female rats. For example, Astrom et al., (1987) observed that 3-MC treatment (20 mg for five days) increased the UDPGT activity towards NA by 467% in male Sprague-Dawley rats and by 258% in female Sprague-Dawley rats, over their respective controls (Table 4.5). Moreover the control UDPGT activities between the two sexes were significantly different, 7.4 ± 0.4 nmol NA glucuronide formed $min^{-1}mg^{-1}$microsomal protein for male rats and 5.3 ± 0.3 nmol NA glucuronide formed $min^{-1}mg^{-1}$microsomal protein for female rats ($p < 0.001$).

Although the induction with PB towards HP-UDPGT was significant in the present study, PB does not seem to be an effective inducer. In this work, female Sprague-Dawley rats were used for experiments. This may be one of the factors for a relatively lower percentage

stimulation for the HP specific enzyme activity compared to the values reported in the literature. Furthermore, the number of animals used in all the studies discussed was fairly small compared to the present study (Table 4.5), suggesting ambiguities in the reported values.

In conclusion, it can be stated that UDPGT enzyme form that preferentially glucuronidates NA also glucuronidates HP and zearalenone as indicated by Substrate Stimulation and Induction studies. These two studies also indicate that the UDPGT enzyme form responsible for HP also glucuronidates NA and zearalenone to some extent. Both the prototypes of inducers, 3-MC and PB significantly induce zearalenone glucuronidating activity. Thus overlapping substrate specificities for both the GT forms is indicated. At the same time, there is no conclusive evidence regarding the heterogeneity of UDPGT in the present work.

4.3 METABOLISM STUDY

Conjugation of zearalenone or its metabolites by the addition of glucuronic acid deactivates the compound. The newly formed conjugates have decreased membrane permeablity and increased water solublity, thereby facilitating renal clearance of the substrate.

Conjugation and urinary excretion limit enterohepatic circulation of zearalenone and may act to limit its toxic expression (Kiritsy et al., 1987, Fitzpatrick et al., 1988a).

In the metabolism study, the urinary excretion of conjugated α-zearalenone was significantly more in the PB administered animals (9.9µg) compared to the controls (5.5µg) ($p<0.05$) (Table 4.6). While the amount of conjugated zearalenone increased 22% from 38.7 to 47.6 µg, the response was too variable to demonstrate significance. The total zearalenone recovered in the urine of the two groups of animals is not significantly affected with 201 µg and 226 µg recovered in the control and induced group of animals respectively.

In this study, 32-36% of the total dose administered was recovered in the urine. This reconfirms the previous studies done in our laboratory (Fitzpatrick et al., 1988a and Kiritsy et al., 1987) which are in contrast to Smith (1982) (Details given in Table 2.1). In a study to determine the effect of dietary protein on zearalenone metabolism and toxicity in the rat, Fitzpatrick et al., (1988a) observed that dietary protein enhanced urinary excretion of zearalenone and its metabolites. The rats received 1 mg/kg body weight zearalenone by stomach intubation and for the 15% dietary protein animals, 26% of the oral dose was

TABLE 4.6 Metabolic study. Effect of phenobarbital induction on 96-h urinary excretion of conjugated zearalenone and α-zearalenol.+

	Control	Pretreated
Free zearalenone	143.0 ± 13.1	153.9 ± 15.4
Conjugated zearalenone	38.7 ± 7.8	47.6 ± 10.9
Free α-zearalenol	13.4 ± 1.4	14.2 ± 1.6
Conjugated α-zearalenol	5.5 ± 0.9	9.9 ± 1.8*

Each value is expressed in μg and represents the mean ± MSE of 12 and 11 animals for the control and the experimental group of animals respectively.

+ Total recovery for the control group was 201μg, 32% of oral dose. Total recovery for the pretreated group was 226μg, 36% of oral dose.

* Significantly different from control ($p<0.05$).

recovered in the urine. Conjugated urinary zearalenone and α-zearalenol constituted 10.3% of the total urinary zearalenone while α-zearalenol, both free and conjugated constituted 5.6% of the total dose recovered in the urine.

Kiritsy et al., (1987) studied the effect of reduced feed intake on zearalenone metabolism in female rats. The animals were orally dosed with 1 mg/kg body weight zearalenone and 33% of the total dose was recovered in the urine in the control group of animals. There were no conjugated zearalenone and its metabolites found in the feces. Conjugated zearalenone and its metabolites constituted 25.6% of the total urinary zearalenone recovered, while α-zearalenol, both free and conjugated, constituted 10.7% of the total urinary zearalenone recovered in the control group of animals. These values are comparable with the values reported in the present study.

Smith (1982) observed the effects of dietary protein alfalfa and anion-exchange resin on excretory metabolites following an oral dose of 10 mg/100g body weight zearalenone. Recovery in the urine was only 3% of the total dose administered. Conjugated urinary zearalenone and metabolites were found only in the urine and were detected to be 28% of the total urinary recovery while α-zearalenol, both free and conjugated

was 12.2% of total urinary recovery for zearalenone. The percentages of conjugated and reduced metabolites reported in this study are comparable to our results but the percentage of urinary recovery itself is extremly low. This may be the result of overdosing of the animals (Fitzpatrick et al, 1988a).

In a [^{3}H] labelled study to determine the excretion profile of zearalenone and its metabolites in rats, the same researcher (Smith, 1980) detected 31.1% of the total dose in the urine. Quantification of the metabolite forms was not carried out in this experiment.

Mirocha et al., (1980) observed that rats given dietary zearalenone (5 mg/twenty-one day old rat) excreted zearalenone principally in free and conjugated forms while only a trace of α-zearalenol was found. Total metabolites excreted in the urine constituted on an average 30% of the total dietary dose which agrees with the value reported in the present study.

There is no agreement in the literature about the percentage of administered zearalenone dose excreted in the urine. Ftizpatrick et al., (1988b) have suggested that the discrepancies in the literature may be due to differences in the recoveries.

More zearalenone was metabolized by conjugation than by reduction (23% and 10% of the total dose recovered

respectively). Kiessling and Petterson, (1978) observed that the amount of conjugated zearalenone and its metabolites was two to five times more than reduced zearalenone.

Conjugation is a deactivation reaction (Caldwell, 1982). Fitzpatrick et al., (1988a) and Kiritsy et al., (1987) have suggested that the conjugation of zearalenone and its metabolites acts to reduce the toxic expression of zearalenone by increasing urinary excretion thus limiting enterohepatic recirculation (Smith, 1980). Administration of inducer increases hepatic zearalenone conjugating activity thereby increasing the amount of deactivated zearalenone.

Alpha-zearalenol constituted more than 10% of the total zearalenone recovered in the urine. The importance of a significant increase in the conjugation of α-zearalenol due to inducer treatment observed in our study, lies in the fact that α-zearalenol is the most potent zearalenone metabolite (Picken et al, 1989). Increased conjugation of the metabolite would reduce toxicity of a zearalenone dose.

CHAPTER V

CONCLUSION

This is the first study that has attempted to characterize the UDPGT isoenzyme responsible for zearalenone glucuronidation in the rat liver and to identify a metabolic inducer, responsible for the induction of UDPGT enzyme activity towards zearalenone.

In the Substrate Stimulaton assays, the activity of rat liver UDPGT towards zearalenone was inhibited by both 1-naphthol, a GT_1 substrate and 4-hydroxybiphenyl, a GT_2 substrate. Pretreatment of animals with the inducers 3-methylcholanthrene (GT_1 inducer) and phenobarbital (GT_2 inducer) increased UDPGT activity towards zearalenone, 1-naphthol and 4-hydroxybiphenyl. Thus it appears that both the isoenzymes GT_1 and GT_2 that have been unambiguously characterized in the literature have overlapping substrate specificities and that both are responsible for zearalenone conjugation. No evidence was seen to support the theory of heterogeneity of UDPGT isoenzymes.

Hepatic microsomal enzyme induction has been known to increase detoxicifation in mammals. In the present study, as

a result of UDPGT enzyme induction by phenobarbital, more conjugated and there by detoxified zearalenone was excreted in the urine. Also, the excretion of conjugated α-zearalenol was significantly increased. Since α-zearalenol is approximately ten-folds more potent than zearalenone, the increased conjugation may help reduce zearalenone toxicity.

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APPENDIX A

UDP GLUCURONYL TRANSFERASE ASSAY

(i) Method Development:

The method by Mulder and Van Doorn (1975) was followed in the present work. Validation of the method was carried out to determine that enzyme system was saturated and that the protein concentration used was appropriate. After testing, it was concluded that the method was working at optimum conditions. The final concentration of the acceptor substrate was reduced to 0.15 M in our modifications.

(ii) Principle:

$$\text{R-OH} + \text{UDP glucuronate} \xrightarrow[\text{transferase}]{\text{UDP glucuronyl}} \text{R-O-glucuronide} + \text{UDP} \qquad \text{(A.1)}$$

$$\text{UDP} + \text{phosphoenolpyruvate} \xrightarrow[\text{kinase}]{\text{pyruvate}} \text{UTP} + \text{pyruvate} \qquad \text{(A.2)}$$

$$\text{Pyruvate} + \text{NADH} + H^{+} \xrightarrow[\text{dehydrogenase}]{\text{lactate}} \text{lactate} + NAD^{+} \qquad \text{(A.3)}$$

The assay is based on measurement of UDP production during the glucuronidation reaction (A.1) which is linked by reactions (A.2) and (A.3) to the conversion of NADH into NAD^{+}. This conversion can be continuously monitored spectrophotometrically at 340 nm because the reduced nicotinamide-adenine dinucleotide $NADH_2$ absorbs light with a peak wavelength at 340nm, while the oxidized form, NAD^{+} shows no absorption between 300nm and 400nm. Thus any conjugation reaction in which $NADH_2$ is oxidized can be measured by monitoring the decrease of absorbance at 340nm. High lipid solubility is a prerequiste for a high rate of glucuronidation.

(iii) Homogenate Preparation:

Livers from female rats were excised, weighed and chilled on ice. Two grams of liver was cut into small pieces with scissors and homogenized in a glass homogenizing tube with a Teflon pestle with 15ml of ice cold 0.154M KCl solution. The sample was homogenized for 1 minute on ice. The homogenate was centrifuged at $4^{0}C$ for 20 minutes at

2000g and the supernatant was extracted with a pipet to exclude the fat layer and kept on ice.

The protein concentration of the supernatant was measured using the method described by Lowry (1951). The supernatant was then diluted with 0.1% Triton X-100 (W/V) in 0.154M KCl solution in order to adjust its protein concentration from the original 12-16mg/ml to a concentration of 0.7-0.9mg/ml.

(iv) Preparation of the Assay medium:

The assay medium containing (per 100ml), 95ml of 0.105M tris buffer, pH 7.3 (the Tris solution was adjusted to pH 7.3 with conc. HCl), 0.1352g $MgCl_2$, 0.0056g phosphoenol pyruvate, 0.0210g NADH, 0.0012g pyruvate kinase (540U/mg), 0.017ml lactate dehydrogenase (5000U/ml), 5.0ml of 4.0 mM acceptor substrate (dissolved in ethanol:H_2O, 1:1) and 0.1346g ammonium UDP glucuronic acid was prepared fresh daily. In the blanks, UDP glucuronic acid was left out of the incubation medium.

(v) Assay:

The assay medium and their respective blanks in the volumes of 1.5ml were placed in 10 mm-light path cuvettes and incubated simultaneouly in a 30^0C water bath for 5 minutes. After incubating the reaction was started by the

addition of 0.5ml of the diluted supernatent preparation and the contents were mixed. The cuvettes were immediately placed in spectrophotometer where the temperature of circulating water bath was 36^0C. The change in the absorbance at 340nm in a five minutes time interval was monitored.

(vi) Final Concentrations in the Assay Medium:

The final concentrations in the assay medium were as follows :

75.0 mM - Tris-HCl buffer

5.0 mM - $MgCl_2$

0.2 mM - phosphoenol pyruvate

0.2 mM - NADH

1.5 mM - UDP glucuronic acid

0.15 mM - acceptor substrate

5.0 U/ml - pyruvate kinase

0.625 U/ml - pyruvate kinase

0.023% - Triton

0.1875-0.2125 mg/ml of the diluted homogenate.

APPENDIX B

HPLC ANALYSIS FOR UDPGT ACTIVITY

(i) Method Development:

Modifications were made to the Mulder and Van Doorn (1975) method for UDPGT enzyme assay. Samples were extracted and run on HPLC.

(ii) Homogenate Preparation:

As described in Appendix A. The protein concentration was adjusted to 2mg/ml using 0.154M KCL buffer containing 0.1% Triton.

(iii) Preparation of the Assay Medium:

The assay medium contained 0.0335g of UDP ammonium salt, 0.00762g of D-Saccharic acid 1,4-Lactone and 1ml of $MgCl_2$ solution (0.0338g/ml $MgCl_2.H_2O$ in water) dissolved in 0.105M tris Buffer, pH 7.3 (Tris buffer was adjusted to pH 7.3 with concentrated HCl) and diluted to 25ml.

(iv) Assay:

A 0.6ml assay medium was pipeted into 5ml test tubes and placed in 37^0C shaking water bath.After 5 minutes 0.03ml of 4mM substrate was added and vortexed for 30 seconds. To each of the tube, 0.2ml of homogenate (2mg/ml) was added and mixed for 20 seconds. After exactly 10 minutes, 100µl aliquot was removed from the test tube and transfered to an 8ml screw-top tube with a teflon lined cap containing 2ml of 10% propanol in ether. The tubes were mixed for 20 seconds. Similar aliquots were taken at 20 and 50 minutes.

(v) Extraction Procedure:

After the assay, 2ml of distilled water was added to each tube and vortexed for 30 seconds. The samples were centrifuged at full speed (1500g) in CS centrifuge for 2 minutes. The top ether layer was transfered to a clean 8ml screw-topped tube using a pasture pipet. A 2ml of 10% propanol in ether was added to the aqueous layer and the extraction procedure was repeated. The two extracted ether layers were then combined. Ether was evaporated to dryness under nitrogen in a $35-40^0$C water bath. A 5ml diluting solvent was added to the tubes and was vortexed for 30 seconds.

(vi) Mobile Phase:

Water : Methanol : Acetonitrile and Phosphoric acid (33:42:15 + 0.2%). Water, methanol and acetonitrile were HPLC grade. Flow rate 1.0ml/minute.

(vii) Standards:

Zearalenone and α-zearalenol (International Minerals Chemicals Corp., Terre Haute, IN).

APPENIDX C

HPLC ASSAY FOR ZEARALENONE AND METABOLITES

(i) Method development:

Analysis of urine was carried out based on the modifications to the Trenholm et al., (1981) made in the laboratory earlier (Fitzpatrick et al., 1987). The flow rate was 1ml/minute and standards contained 50ng of zearalenone and 50ng of α-zearalenol. Adjustments were made to the mobile phase to reduce the peak separation.

(ii) Hydrolysis and Extraction:

A 0.1ml urine sample and 1.8ml glass distilled water were pipetted into an 8ml screw-top test tube. A β-glucuronidase solution (Type B-1 III Sigma Chemical Co.), 0.1ml, in glycerol was added. Tubes were loosely capped and the mixture was incubated overnight at 37^0C in a water bath. Two mls of 10% 2-propanol in ether was added to the cooled incubated tubes. The mixture was vortexed, layers allowed to separate and the top layer was removed. This step was repeated. The two extraction layers (ether) were combined. The ether layer was placed on ice for 10-15 minutes, and adjusted to pH 12 with the addition of 2.0ml of chilled

0.184 M NaOH. The mixture was vortexed and then chilled on ice. The layers were separated and the top layer was discarded. The cold acqueous layer was washed with 2ml portions of benzene. To avoid the formation of an emulsion the tubes were gently inverted while mixing. The samples were chilled and the top (benzene) layer was discarded. This step was repeated. Phenolphathalein indicator (3 drops) was added and the sample was neutralized to pH 7.0-8.0 range by adding 0.5N chilled acetic acid. The indicator turned from red to slightly yellow. The sample was chilled and extracted 3 times with 2.0ml of benzene, vortexed 30 seconds and chilled. The extracts were combined and evaporated to dryness. Samples were taken up in an known volume of the mobile phase without phosphoric acid. For the determination of free zearalenone and α-zearalenol in urine, the incubation step was omitted and 1.9ml of water was added to the sample. Standards of unknown concentration were used to establish that the recoveries from the hydrolysis, clean up and extraction were quantitative.

(iii) Mobile Phase:

Water : Methanol : Acetonitrile and Phosphoric acid (40:48:12 + 0.2%). Flow rate was 1.0ml/minute.

(iv) Standards:

Zearalenone and α-zearalenol (International Minerals Chemical Corp., Terre Haute, IN).

APPENDIX D

MATERIALS

Magnesium Chloride - BDH Chemicals Ltd., Poole England.

Phosphoenol Pyruvate - Sigma, St. Louis, Missouri, U.S.A.

Nicotinamide Adenine Dinucleotide (reduced form) - Sigma.

Pyruvate Kinase - Sigma.

Lactate Dehydrogenase - Sigma.

Uridine Diphosphate Glucuronic Acid - Sigma.

1-Naphthol - Sigma.

4-Hydroxybiphenyl - Sigma.

Zearalenone - gift of IMC Terre Haute, Indiana, U.S.A.

Bovine Serum Albumin - Sigma.

Phenobarbital (Sodium Injection USP) 30mg/ml

- Abbott Laboratories Ltd., Toronto plant, Canada.

3-Methylcholanthrene - Sigma.

β-Glucuronidase- Sigma.

APPENDIX E

LIST OF MAJOR EQUIPMENT

1. Sp6-300 Spectrophotometer, Pye Unicam
2. Sp8-400 UV/VIS Spectrophotometer, Pye Unicam
3. International Centrifuge, International Equipment Co.
4. International Refrigerated Centrifuge, International Equipment Co.
5. Meyer N-Evap Analytical Evaporator
6. Caramo Stirrer Type R2R1-64 Homogenizer, Canlab Polytron
7. Water-bath Shaker, Eberbach Corp.
8. HPLC-Pump, Beckman 110A
9. Whatman CO: PELL ODS Precollum, Terochem
10. Ultrasphere ODS AnaYtical Colum, Beckman
11. Beckman 160 Absorbance Detector
12. Bausch and Lomb VOM 7 Recorder, Fisher Scientific.