Effects of Dietary Protein on Hepatic and Extrahepatic Drug Metabolizing Enzymes

By

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EFFECTS OF DIETARY PROTEIN ON HEPATIC AND EXTRAHEPATIC DRUG METABOLIZING ENZYMES

BY

PRITI BAIJAL

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

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ABSTRACT

Biotransformation of lipophilic xenobiotics to hydrophillic metabolites is catalyzed by phase I and phase II enzyme systems. Dietary protein can alter the rate of this biotransformation. While hepatic enzyme systems have been extensively studied, little information is available on the effect of protein status on extrahepatic systems. The objective of this study was to examine the effect of dietary protein simultaneously on hepatic and extrahepatic tissue. The study also examined the relationship between dietary effects and temporality (duration of protein diets). Forty-eight weanling male Sprague-Dawley rats were fed a low (7.5%), standard (15%), or high protein (45%) diets for 7 or 14 days *ad libitum*. At the end of the treatment periods, reductase and UDPGT enzyme activities were determined in the intestine, kidney and liver.

The study demonstrated that the response to protein diets was tissue and isoenzyme dependant. For example, hepatic reductase activity decreased significantly with decreasing levels of dietary protein ($p < 0.01$), while in the kidney and intestine it remained unchanged on standard and low protein diets and increased on a high protein diet ($p < 0.01$). UDPGT isoenzyme activities in the liver and the intestine were significantly the greatest on a low protein diet ($p < 0.01$), while, in the kidney they were significantly the greatest on a high protein diet ($p < 0.01$). GT_1 activity was modulated by dietary protein in all three tissues, while GT_2 activity was altered only in the intestine, thereby demonstrating that isoenzymes within a tissue also have unique responses to protein modulation.

Our study examined the relationship between dietary effects and temporality which

has not previously been studied. Duration of feeding of the protein diets significantly affected reductase and UDPGT activities in the intestine ($p < 0.01$) but did not affect it in the kidney and liver. This can be explained by the dynamic nature of the intestinal tissue. That, because of the rapid turnover in the intestine any changes in nutritional status are quickly reflected. The absence of the temporal effects in the liver and kidney suggest that once the enzymes have been established by protein modulation, the effect of protein diets in these tissues will not compound over time for at least 14 days. The decline in reductase activity in the intestine of control animals after 14 days may be an age related effect ($p < 0.01$). The absence of this age related effect on the low and high protein diets suggests an adaptive response by the intestine to changes from adequate protein status.

The unique hepatic and extrahepatic tissue and isoenzyme responses suggest that generalizations regarding the metabolic response to protein diets based solely on hepatic studies are erroneous. Our results support suggestions that extrahepatic tissues are not simply attenuated versions of hepatic systems. The temporal effects observed in our study are a snap shot over a 14 day period. In the absence of supporting evidence, these results are too limited to establish long or short term consequences with any certainty.

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Chapter I

INTRODUCTION

Man is constantly exposed to a variety of noxious chemicals that are collectively termed xenobiotics. Xenobiotics are substances foreign to the body including food additives, drugs, industrial chemicals, insecticides and natural agents such as glycoalkaloids or mycotoxins. With few exceptions, xenobiotics are lipid-soluble, weak organic acids or bases that are not readily eliminated from the body. Therefore, they must be extensively metabolized prior to excretion.

Xenobiotics enter the body when inhaled, ingested or dermally absorbed. The major route of exposure is the oral route where xenobiotics are absorbed along with nutrients via active transport, passive diffusion or lymphatic absorption (Chabbra, 1979). Absorption of toxins through the intestinal lymphatics is of particular significance since this route allows chemicals to be distributed throughout the body without first passing through the liver. The intestinal first pass effect refers to biotransformation during drug absorption and represents an important step of presystemic detoxification (Schwenk, 1988).

Once absorbed, xenobiotics must be transformed to polar, water soluble metabolites prior to excretion. Biotransformations are carried out by the enzyme systems located in the liver and extrahepatic tissues including the intestine, kidney and lungs. These enzymes are located in the cell endoplasmic reticulum and cytosol, and metabolize xenobiotics via oxidative, reductive and conjugative pathways. They are known as the phase I and phase II drug metabolizing enzyme systems.

Phase I reactions change the structure and physiochemical properties of xenobiotics. These transformed compounds often retain bioactivity and may, in some cases, manifest increased reactivity. In contrast, phase II reactions, through conjugation, change both the structure and physiochemical properties of the drug, generally resulting in reduced bioactivity. Whether biotransformation produces a toxic intermediate or results in detoxification depends upon the particular substrate and types of intermediates involved. For example, the common biotransformation reactions, hydroxylation, epoxidation, sulfation and glucuronidation can either detoxify or produce a more toxic product depending on the chemical reactivity and/or the biological activity of the products.

Diet can affect the rate of xenobiotic metabolism altering its toxic expression. For example, the toxicity of many xenobiotics increases in protein deficiency (Hathcock, 1987). The effect of diet on xenobiotic metabolism is therefore an important issue. The interaction between dietary protein and xenobiotic toxicity has been examined extensively in hepatic systems. However, it is clear that detoxifying enzyme systems are not confined to the liver. Phase I and phase II systems have been demonstrated and studied in the lungs (Fouts and Devereux, 1972), kidneys (Ellin et al, 1972) and small intestine (Miranda et al, 1979). However, the physiological significance and control mechanisms for the enzymes at these sites are not well understood. Yang and Yoo (1988) suggested that dietary effects on drug metabolism by extrahepatic tissues is largely unknown and remains to be studied. In addition, Philpot (1991) suggested that extrahepatic detoxification enzyme systems are not simply attenuated versions of hepatic systems and

that they warrant examination. Therefore, the objective of this study was to examine the effect of dietary protein on hepatic and extrahepatic drug metabolizing enzymes.

Chapter II

REVIEW OF LITERATURE

Nutritional Toxicology

Chemical substances foreign to the body including food additives, pesticides, industrial chemicals and natural food products, are collectively termed xenobiotics. The study of the interactions between these foreign substances and living organisms is broadly referred to as toxicology. Adverse or noxious effects in biological systems are produced when the agent or its biotransformation product(s) reach target sites at an appropriate concentration and for sufficient time to produce the toxic manifestations. The occurrence and nature of the response depends on the chemical and physical properties of the agent, the exposure situation, and the susceptibility of the biological system or subject (Klaassen and Eaton, 1991).

Nutritional toxicology is the study of food borne toxicants and the interrelations of xenobiotics with nutrients and nutritional states. This includes the study of the diet as a source of toxicant, the effects of toxicant on nutrients and nutritional processes, the effects of nutrients and nutritional metabolism on toxicant, and the scientific basis for regulatory decisions affecting toxicological safety of dietary components (Hathcock, 1982; Hathcock, 1987).

2.1 Introduction

Once ingested, lipophilic xenobiotics can remain in the body almost indefinitely unless they are converted to polar metabolites. This biotransformation of xenobiotics is

carried out by the drug metabolizing enzyme systems located in the cell endoplasmic reticulum and cytosol in hepatic and extrahepatic tissues. These enzyme systems metabolize xenobiotics and endogenous compounds such as fatty acids by oxidative, reductive and conjugative pathways (Sipes and Gandolfi, 1991).

2.2 Characteristics of Xenobiotic Metabolizing Enzymes

Two major enzyme systems are involved in xenobiotic biotransformation (Sipes and Gandolfi, 1991). The first is known as the mixed function oxidase (MFO), or phase I system. Phase I enzymes act through oxidation, reduction and hydrolytic reactions to render xenobiotics more water soluble and potentially more able to react with conjugating enzymes involved in phase II reactions. It is important to note that phase I reactions may also result in changes in activity, serving to activate or inactivate the parent compound. The second biotransformation system is known as the phase II system. This reaction system consists of a number of conjugating enzymes located in the cytosol and endoplasmic reticulum of the cell. These enzymes catalyze the conjugation of xenobiotics and phase I reaction metabolites with endogenous substances such as glucuronic acid and sulphates. Conjugation makes xenobiotics less toxic, more water soluble and promotes their excretion in the urine, bile and faeces. Biotransformation reactions require energy and consume metabolic intermediates such as NAD and NADPH. Since these reactions compete for substrates of intermediary metabolism, biotransformation reactions can redirect cellular metabolism (Hanninen et al, 1979).

2.2.1 Phase I Reaction: Mixed Function Oxidase

Phase I reactions can be described in the following stoichiometry with the X representing a xenobiotic (Peterson and Holtzman, 1980).

$$X . H + O_2 + NADPH \text{ ----------} \blacktriangleright X . OH + H_2O + NADP^+$$

The reaction requires molecular oxygen and two electrons. The enzyme NADPH cytochrome c reductase transfers reducing equivalents from NADPH to the terminal oxidase cytochrome P-450.

A prime function of phase I reactions is to expose and/or add functional groups such as -OH, -SH, $-NH_2$, -COOH to the parent compound, predisposing the compound to undergo phase II reactions. Functional groups are added via two oxidative enzyme systems: the cytochrome P-450 (C-P450) and the mixed function amine oxidase system.

Of the two oxidative enzyme systems, C-P450 system is the most important. It has three components, C-P450, NADPH C-P450 c reductase and a phospholipid embedded in the phospholipid matrix of the endoplasmic reticulum (Coon et al, 1973; Nebert et al, 1981) (Figure 1). The term P-450 is applied to a hemeprotein structurally embedded within the membrane of the endoplasmic reticulum. The hemeprotein functions as a MFO or oxygen transfer agent. NADPH C-P450 c reductase is an enzyme

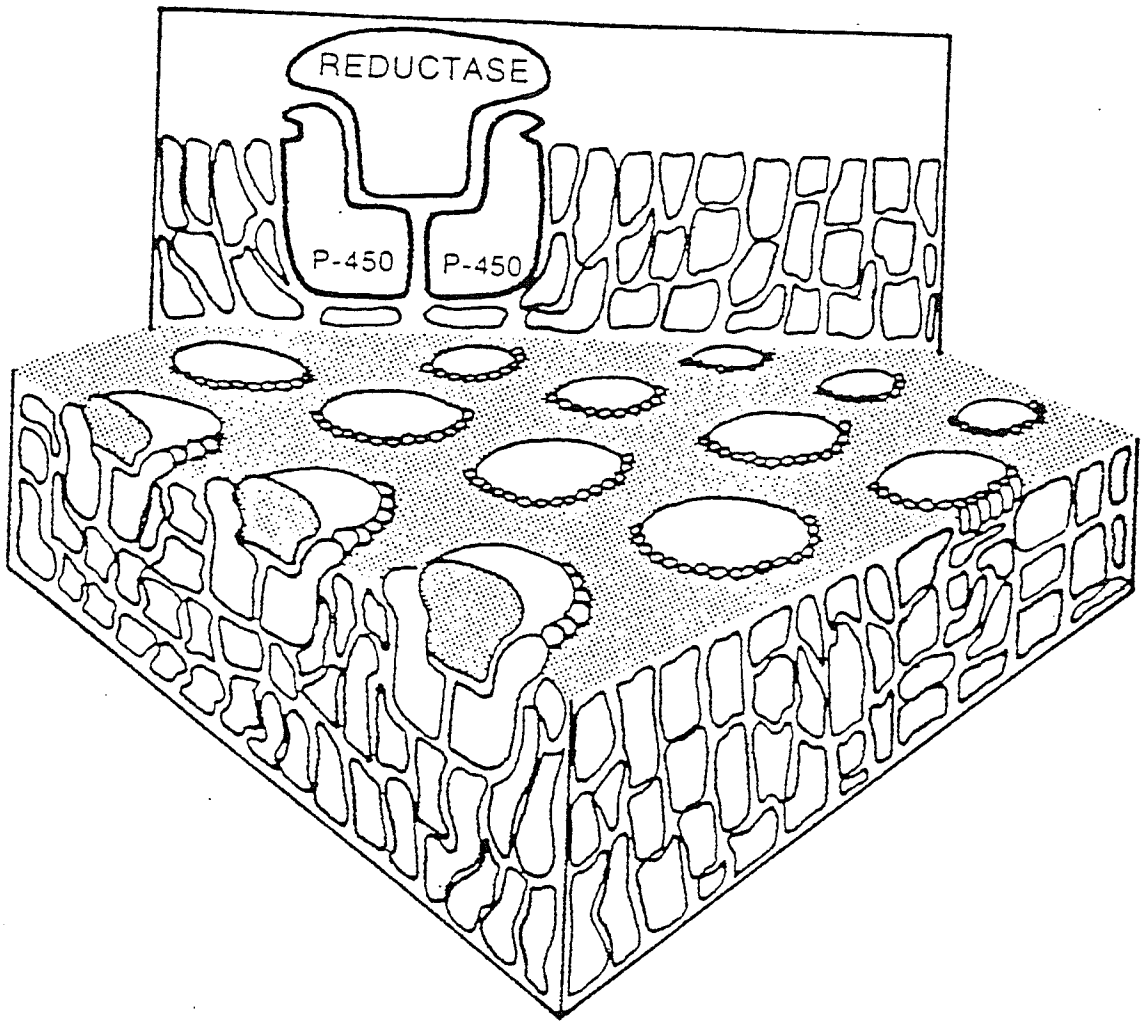


Figure 1: Schematic demonstration of the interaction of C-P450, reductase and lipid (Nebert et al, 1981).

containing FAD and FMN as cofactors. It transfers reducing equivalents from NADPH to C-P450 and it is required for optimal interactions between the two proteins (Coon et al, 1973). The phospholipid is usually phosphotidylcholine and is essential in the transfer of electrons from NADPH to C-P450 (Strobel et al, 1970; Coon et al, 1973). A detailed description of C-P450 mediated reactions is described in Figure 2.

Step 1. The initial step in C-P450 mediated mixed function oxidase is the binding of the substrate to the oxidized form (Fe^{3+}) of Cytochrome P-450.

Step 2: NADPH C-P450 C reductase then transfers an electron from NADPH to the oxidized (Fe^{3+}) P-450 substrate complex reducing it to a (Fe^{2+}) complex.

Step 3: The reduced C-P450 substrate complex then reacts with oxygen to form an oxycytochrome P-450 complex. The oxycytochrome P-450 formed may decompose to form superoxide (O_2^-) and ferric cytochrome P-450 (Estabrook and Weringloer, 1977). The superoxide dismutates to form hydrogen peroxide and oxygen. The formation of superoxide aborts the C-P450 mediated oxidation of drugs.

Step 4: Alternatively, the oxy-ferrocytochrome P-450 substrate complex can undergo a second reduction to form an oxene derivative (Ulrich and Staudinger, 1969) by releasing water. The oxene then hydroxylates the substrate making it more water soluble and regenerates the oxidized form of C-P450.

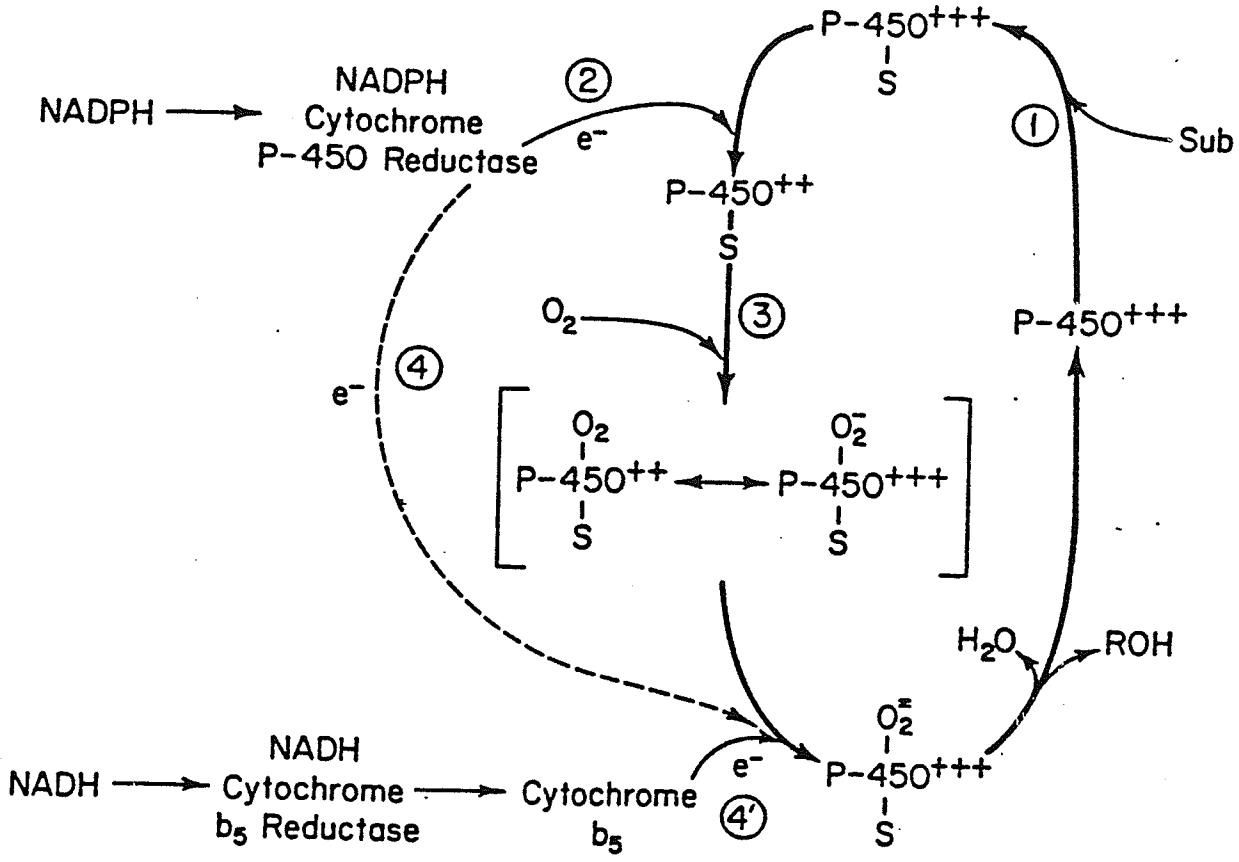


Figure 2: Basic schemes involved in cytochrome P-450 mediated mixed function oxidations (Peterson and Holtzman, 1980).

2.1.2 Phase-II reactions

Phase II reactions are biosynthetic in nature. Enzymes such as sulfotransferase, UDP-Glucuronyltransferase (UDPGT) and glutathione-S-transferase conjugate xenobiotics with endogenous cofactors such as sulfates, glucuronic acid or glutathione resulting in the formation of harmless end products (Parke and Ioannides, 1981; Olson et al, 1992). Phase II reactions are therefore often detoxification reactions.

UDPGT conjugations are quantitatively the most important phase-II reactions (Dutton, 1981). UDPGT catalyzed reactions do not necessarily occur secondary to C-P450 catalyzed phase I reactions. For example, xenobiotics such as morphine, naphthols and phenols exist in the hydroxylated state and serve as substrates for UDPGT without undergoing phase I hydroxylation (Tephly and Burchell, 1990). In addition to xenobiotics, UDPGT inactivates a variety of endogenous compounds such as bilirubin, steroid hormones and thyroxin (Roche et al, 1954; Bidlack et al, 1986).

UDPGT

UDPGT transfers glucuronic acid from uridine diphosphate glucuronic acid (UDPGA) to the xenobiotic (XOH) yielding a glucuronide (Dutton, 1981). UDPGA availability is regulated by the presence of glucose, energy (UTP), and NAD^+ (Dutton, 1981). Through the glucuronic acid pathway, glucose-1-phosphate reacts with UTP to form UDP-Glucose, which is then oxidized to UDPGA utilizing two NAD^+ (Strominger et al, 1954).

XOH + UDP-glucuronic acid ----------▸ XO-glucuronide +UDP

Glucuronides are water soluble, more readily excreted than the parent compound and, in most cases, possess little or no biological activity (Anders, 1980; Olson et al, 1992). However, some metabolites such as glucuronide conjugates of retinoic acid, and morphine are more active than the parent compound (Abbot and Palmour, 1988; Olson et al, 1992). For example, morphine 6-O-glucuronide, a conjugation product of morphine has been shown to be a more potent analgesic than morphine itself (Abbot and Palmour, 1988).

While most phase II enzymes are cytosolic, UDPGT is located in the endoplasmic reticulum, a location that affords UDPGT direct access to the products of microsomal C-P450 enzymes (Sipes and Gandolfi, 1991). This highly integrated microsomal membrane system results in the sequestration of the highly lipophilic compounds, the addition or unmasking of a functional group, and the conjugation of this functional group with the highly polar glucuronic acid moiety (Sipes and Gandolfi, 1991).

UDPGT Isoenzymes

Several distinct forms of UDPGT have been identified. Bock et al (1979) reported on the separation and purification of two UDPGT's from rat liver microsomes, a GT_1 form, which catalyzed p-nitrophenol glucuronidation and a GT_2, form which catalyzed morphine, 4-hydroxybiphenyl and chloramphenicol glucuronidation. Since the first report, purification, chromatofocusing and immunochemical studies have now

provided evidence for a minimum of eleven isoenzymes of UDPGT (Sipes and Gandolfi, 1991).

Tephly et al (1988) have suggested that endogenous UDPGT substrates are helpful in distinguishing UDPGT isoenzymes. For example, endogenous substrates such as steroids react with only a single isoenzyme, whereas certain xenobiotic substrates such as p-nitrophenol react with several enzyme forms. They suggest that UDPGT isozymes are relatively specific for endogenous substrates such as steroids, but have may broad substrate specificities for xenobiotic substrates.

2.2.3 Relationship Between Phase I and Phase II Reactions

The relationship between phase I and phase II reactions is summarized in Figure 3. Metabolic fate of a compound is determined by its physical/chemical products. For example, some volatile organic compounds may be eliminated through the lungs with no biotransformation. Compounds with functional groups may be conjugated directly, whereas others undergo phase I reactions before conjugation (Sipes and Gandolfi, 1991). While, both phase I and phase II enzymes are referred to as detoxification enzymes, it must be emphasized that biotransformation is not strictly related to detoxification. In a number of cases, the metabolic products are more toxic than the parent compound. This is especially true for some carcinogens, organophosphates, carbontetrachloride (CCl_4) and paracetamol (Sipes and Gandolfi, 1991). For example, Slater (1966) demonstrated that CCl_4 is converted to a highly reactive free radical CCL_3 and paracetamol is metabolized to a toxic quinoneimine (Miner and Kissenger, 1979).

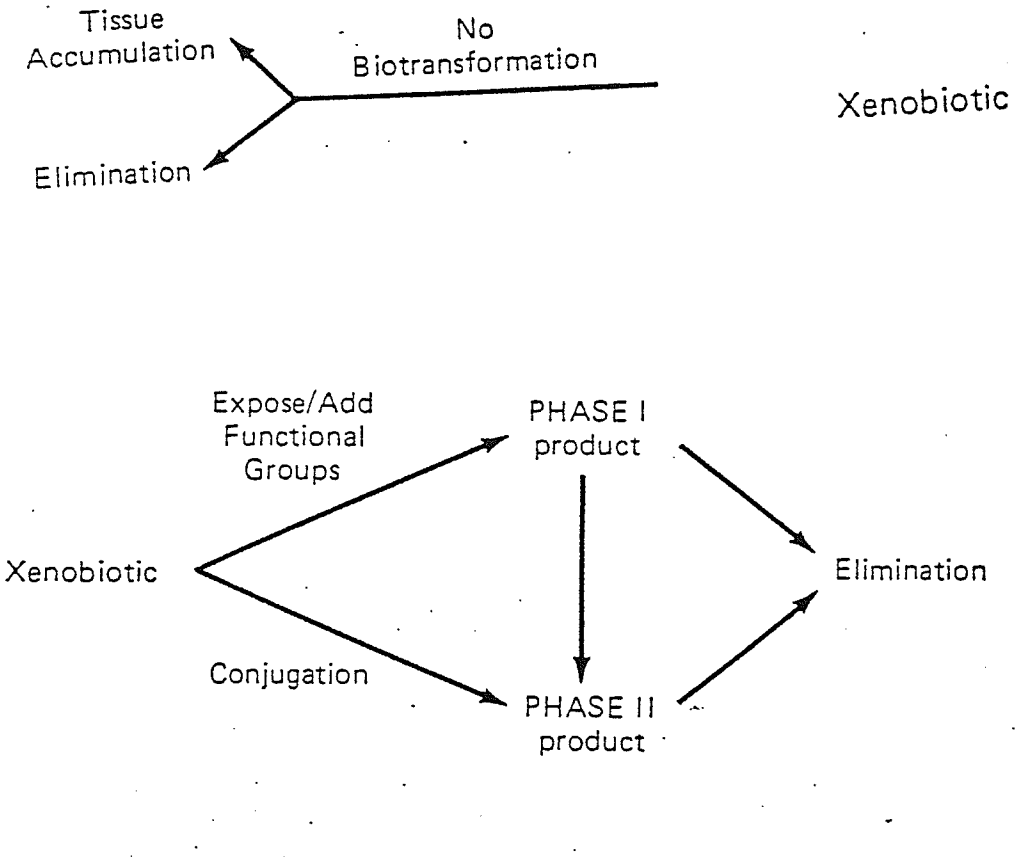


Figure 3: Integration of phase I and phase II biotransformation reactions (Sipes and Gandolfi, 1991).

2.3 Factors Affecting Biotransformation of Xenobiotics

Biotransformation of xenobiotics can be influenced by a number of factors including age, sex, species, genetics, diet and induction.

The ability of animals to perform biotransformation reactions is extremely low in the early foetus and enzyme activity does not reach maximal levels until the peri or post natal period (Hanninen et al, 1979). In rats, C-P450 activity is low at birth but develops rapidly reaching maximal activity by thirty days of age (Sipes and Gandolfi, 1991). The UDPGT isoenzyme forms GT_1, GT_2, and GT_3 appear sequentially during fetal development in the rat (Bock et al, 1979). In the case of UDPGT, activity towards bilirubin reaches adult levels by five to seven days (Sipes and Gandolfi, 1991).

C-P450 and reductase concentrations are 20-30 % higher in male hepatic microsomes as compared to females (Sipes and Gandolfi, 1991). In general, male rats but not male mice, tend to metabolize compounds more rapidly than females in both hepatic and extrahepatic tissue (Gangolli and Phillips, 1988 ; Sipes and Gandolfl, 1991). Glyceryl guaiacolate ether, a centrally acting muscle relaxant, has a shorter circulating half life in male rats compared to female rats because of higher O-demethylase activity in males (Giri, 1973).

Hassen (1993) demonstrated that postnatal development pattern of UDPGT activity is substrate, gender and age specific. Enzyme activity towards GT_1 substrates (zearelenone and α- naphthol) is greater than toward GT_2 substrates (p-hydroxy biphenyl) in all age groups. Immature female rats (35 days old) exhibited a greater capacity for hepatic conjugation than males of a similar age, while adult male rats (112 days old)

exhibited greater UDPGT activity than females of a similar age. The age dependant decrease was gender specific, with the decline in females more pronounced than in males.

Animal species differ in their biotransformation capabilities (Hanninen et al, 1979). For example, 2-hydroxylation of biphenyl, is high in mice and low in rats, and monobutylphthalate glucuronidation is high in hamsters and low in rats (Gangolli and Phillips, 1988).

While, individual biotransformation ability is genetically influenced, enzyme induction in response to environmental chemical challenges may cause variation within wide limits (Hanninen et al, 1979). Enzyme activity increases in response to a variety of challenges (Sipes and Gandolfi, 1991). This increase in activity is due to an increase in enzyme protein and is referred to as enzyme induction. This adaptive response, often leads to increased elimination and detoxication of the xenobiotic, inducer and other related compounds (Bock et al, 1990). However, induction sometimes also leads to increased toxicity for example, benzo(a)pyrene mutagenicity (Bock et al, 1984 ; Bock et al, 1990).

In hepatic tissue, C-P450 and UDPGT are induced by phenobarbital (PB) and the polycyclic aromatic hydrocarbons benzo(a)pyrene (BP) and 3-methylcholanthrene (3MC) (Sipes and Gandolfi, 1991). C-P450 isoenzymes induced by PB, BP and 3MC are virtually absent in liver microsomes of untreated animals (Sipes and Gandolfi, 1991). In extrahepatic tissues, C-P450 and UDPGT enzymes are not readily induced by PB and compounds that produce a similar pattern of induction in the liver (Hanninen and Aitio,

1968; Sipes and Gandolfi, 1991). Polycyclic aromatic hydrocarbons induce C-P450 in extrahepatic tissue such as lungs, intestine, kidney and skin (Sipes and Gandolfi, 1991). Compounds such as salicylic acid increase UDPGT activity in extrahepatic tissue (Hanninen and Aitio, 1968). It has been postulated that the inducible enzyme systems at these entry portals serves as a defence mechanism (Sipes and Gandolfi, 1991).

2.4 Effect of Diet on the Metabolism of Xenobiotics

There has been a growing recognition of the importance of nutritional status on the metabolism of xenobiotics since the initial observations of Mueller and Miller (1950). Deficiencies of nutrients such as protein, lipids (essential fatty acids), vitamins and minerals can reduce detoxification processes and act to increase the toxic expression of these compounds (Hathcock, 1982). Hathcock (1982) suggested that microsomal C-P450 dependant MFO is especially sensitive to nutritional deficiencies. The MFO is dependant on a continuous supply of NADPH generated by other enzyme systems which, are regulated by their respective cofactor supplies (Thurman and Kauffman, 1980). Nutrition can have profound effects on cofactor supply, thereby influence xenobiotic toxicity by modification of monooxygenase and conjugation reactions.

Nutritional deficiencies also decrease biochemical and structural integrity of cells increasing their susceptibility to toxin damage. In addition, deficiencies decrease the rate of cellular replacement, enzyme synthesis and activation, and coenzyme synthesis, factors which increase cell susceptibility to toxins (Wattenberg, 1975). Thus, nutritional deficiencies alter the primary susceptibility of cells to toxicants (Hathcock, 1982).

2.4.1 Protein Deficiency

Protein deficiency may either exacerbate or alleviate the action of chemical compounds depending on the nature of the parent compound (Hathcock, 1987). Therefore, when judging the possible role of dietary protein in terms of toxicity of a particular compound, one must consider whether it is the parent compound or the metabolite that is the active agent *in vivo*:

1) A detoxification reaction is one in which product is less reactive than the parent compound. In such a reaction, protein deficiency would increase the observed toxicity by reducing metabolism. Many pesticides, such as the chlorinated hydrocarbons, are examples of compounds showing increased toxicity during protein deficiency (Boyd and Krupa, 1970).

2) However, when the parent compound is metabolized to a more toxic product, protein deficiency may decrease the observed toxicity. For example, protein deficiency decreases carbon tetrachloride toxicity (McLean and McLean, 1966).

2.4.2 Public Health Concerns

Protein deficiency is a common problem in the developing world and represents a public health problem in many countries. Protein deficiency also increases man's vulnerability to toxicological insults by xenobiotics due to altered rates of metabolism of drugs and foreign compounds (Boyd and Krupa, 1970; Krijnen and Boyd, 1971; Campbell and Hayes, 1976; Mandel et al, 1992).

Khanna et al (1992) studied toxicity and nutritional status during pregnancy

suggesting that inadequate protein intake would make pregnant mothers vulnerable to noxious agents. They investigated the effects of protein malnourishment on acrylamide (a vinyl monomer, highly reactive, that has a wide variety of industrial applications) toxicity in pregnant and nonpregnant Wistar rats. On day 1 of gestation, pregnant rats were fed either a low (8% casein), or, a normal protein diet (20% casein) until 17 days of gestation. On day 6 of gestation, half of the rats were administered 2-10 mg acrylamide/kg body weight (BW) until day 17 of gestation. Non pregnant female rats were pair fed either, a low or normal protein for 17 days. Acrylamide was administered as in pregnant rats. Pregnancy under conditions of protein malnourishment modified the susceptibility of rats towards acrylamide. Pregnant rats fed the low protein diet and given more than 2 mg acrylamide/kg BW had a 80% mortality rate, while no deaths were seen in pregnant and nonpregnant rats fed a normal protein diet or in the protein malnourished non pregnant rats. While the mechanism of toxicity is unclear, these observations suggest that we should be concerned about the use of environmental chemicals, particularly in developing countries where nutritional status may be poor (Khanna et al, 1992).

The interactions between dietary protein, xenobiotic metabolism and toxic expression have been examined in hepatic drug metabolizing enzyme systems. However, detoxifying enzyme systems are not confined to the liver. In addition, extrahepatic detoxification enzyme systems are not simply attenuated versions of hepatic systems (Philpot, 1991). Therefore, it is important that both hepatic and extrahepatic metabolism be investigated if we are to understand the relationship between nutritional states and xenobiotic toxicity.

2.5 Hepatic and Extrahepatic Metabolism

Practical considerations such as ease of availability, size, ease of fractionation and suitability for enzyme purification facilitated the study of the liver systems as opposed to those of extrahepatic tissue, which are more difficult to access, fractionate and purify (Philpot, 1991). Recently, phase I and phase II systems have been demonstrated and studied in the lungs (Domin et al, 1983) kidneys (Ellin et al, 1972) and small intestine (Miranda et al, 1979; Pascoe and Correia, 1985). These systems are quantitatively significant, respond to physiological challenges and are inducible by specific xenobiotics.

Boyd and Statham (1983) suggest that when comparing hepatic and extrahepatic metabolism, two basic scenarios must be considered:

I) Liver is the primary site of formation of reactive intermediates. In this scenario:

a) Toxicity occurs primarily in the liver and not in extrahepatic tissues. CCl_4 is an example of a xenobiotic showing such toxicity. CCl_4 toxicity is dependant on activation by the C-P450 system (Timbrell, 1991). Since the liver contains the greatest concentration of C-P450, it is the target organ for CCl_4 toxicity (Timbrell, 1991).

b) Alternatively, reactive intermediates may travel from the liver to extrahepatic tissues via the bloodstream and toxicity occurs both in the liver and at extrahepatic sites. Monocrotaline, a toxic compound of plant origin, is an example of a xenobiotic showing such toxicity. Monocrotaline, after hepatic metabolism, produces a highly reactive pyrrole derivative leading to

hepatotoxicity. The pyrrole derivative can also be delivered via circulation to the lungs and produce pulmonary vascular endothelium necrosis, an example of extrahepatic toxicity.

II) Both the liver and extrahepatic tissue(s) are sites of formation of reactive intermediates. In this scenario:

a) Reactive intermediates may be formed and bound in the extrahepatic tissue or delivered from the liver to the extrahepatic tissue via circulation. Bromobenzene is an example of a xenobiotic showing such toxicity. Metabolism of bromobenzene in the liver and lungs produces an epoxide, a reactive metabolite, which causes hepatic and pulmonary necrosis. The epoxide can also be delivered to the lungs from the liver thus, toxicity occurs at both sites.

b) Alternatively, reactive intermediates may bind only in the tissues where they are formed. This occurs due to the localized presence of specific isoenzymes that are highly efficient in the metabolism of the parent compound. 4-Ipomeanol, a naturally occurring furan derivative, is metabolized in the lung to a highly reactive metabolite causing pulmonary clara cell necrosis. 4-Ipomeanol is also metabolized in the liver; however, the level is insufficient to produce liver necrosis.

2.6 Significance of Extrahepatic Metabolism

Gastrointestinal Tract

The intestine serves as a major entrance route for drugs and xenobiotics. The extensive surface area and rapid turnover of mucosal cells makes the intestine quantitatively important for metabolism and enables it to respond rapidly to dietary modulations or xenobiotic challenges (Gangolli and Phillips, 1988). Xenobiotic metabolism is greatest in the developed enterocytes of the tip cell region, cells which are most active in absorption (Hoensch et al, 1979; Figure 4).

Hoensch and Hartman (1981) suggest that the intestine serves as the body's first line of defence against drugs, carcinogens and other toxic agents through enzymatic biotransformation, thereby reducing the body's toxic burden. The xenobiotics in the small intestine lumen are taken up into epithelial cells and the xenosystem inactivates them by glucuronidation, sulfation or glutathione conjugation (Dawson and Bridges, 1979). The inactive biotransformation products either diffuse back into the lumen or remain within the mucosal cell, and eventually return to the lumen as cells are sloughed due to normal cell migration within the villous structure. Therefore, little of the toxin enters portal circulation as first-pass metabolism prevents many xenobiotics from being distributed throughout the body. An example of intestinal xenosystem metabolism was demonstrated for benzo(a)pyrene (Bock et al, 1979). Thirty minutes after benzo(a)pyrene introduction to the closed intestinal loop, 54% of the dose was recovered in the free form in the lumen and 3% was observed in the portal blood. In addition, 6% of benzo(a)pyrene was recovered as glucuronide metabolites in the lumen and 37% observed in the portal blood.

The jejunal loop system demonstrated extensive first pass metabolism of benzo(a)pyrene.

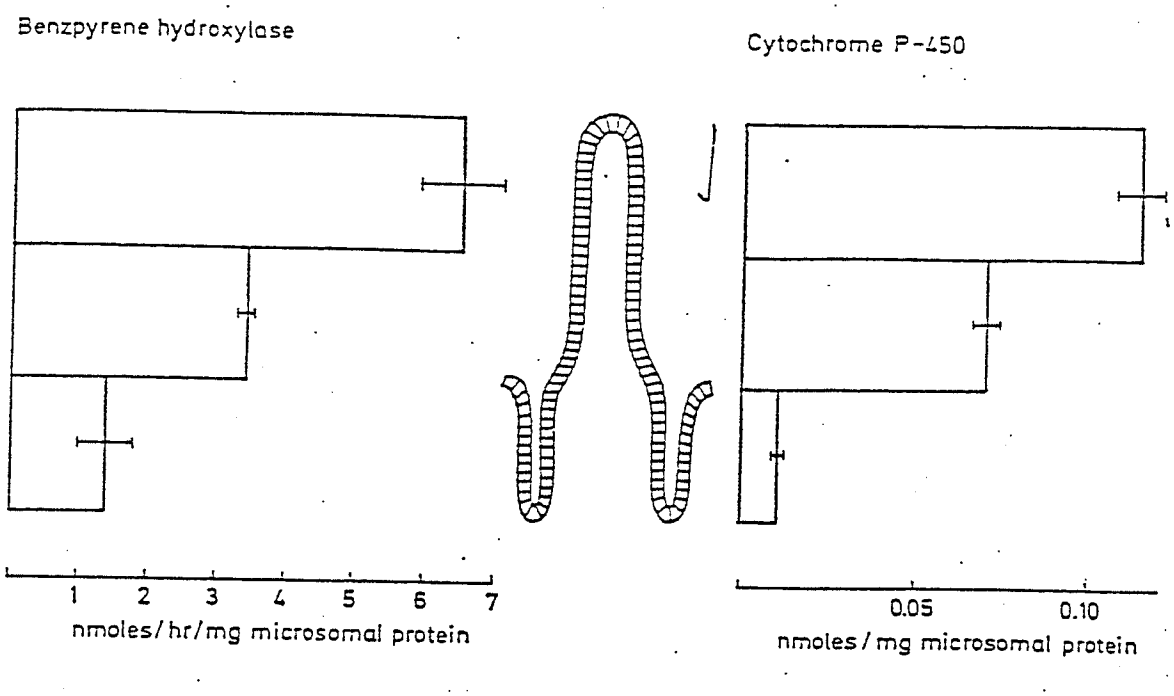


Figure 4: Distribution of C-P450 and Benzpyrene hydroxylase in epithelial cells in duodenal mucosa of rats (Hoensch et al, 1979).

Kidneys

The primary functions of the mammalian kidneys are the excretion of waste products and regulation of blood electrolytes. Although, kidney's role as a xenobiotic excretory organ is well described, its involvement in biotransformation reactions is poorly understood (Anders, 1980). Since, the kidney receives 25% of the total cardiac output, it has the potential to make a significant contribution to the total metabolic alteration of xenobiotics in the body (Anders, 1980 ; Timbrell, 1991). The decrease in water volume during urine passage may concentrate those xenobiotics that did not undergo tubular reabsorption (Dekant and Vamvakas, 1992). Thus, xenobiotics may reach toxic concentrations in the distal parts of the nephron inducing papillotoxicity (Sabatini et al, 1983). Also, compounds such as cephaloridine are actively transported from blood into tubular fluid and may accumulate in the proximal tubular cells at concentrations that could result in proximal tubular damage (Timbrell, 1991). Quantitatively significant C-P450 activity is located in the renal cortex (Anders, 1980) and may be responsible for metabolic activation of chloroform and paracetamol, explaining the renal toxicity of such compounds (Timbrell, 1991). Finally, a variety of xenobiotics form glucuronides in the kidney, and this pathway is important in detoxification (Anders, 1980; Tarloff et al, 1987). The highest UDPGT enzyme activity is observed in the proximal tubule and multiple forms of UDPGT have been identified in the kidney (Hjelle et al, 1986).

2.7 Metabolic Studies Involving Hepatic and Extrahepatic Tissues

The effect of diet on microsomal enzymes involved in the metabolism of xenobiotics has been extensively studied in the liver. Research suggests that the quality and quantity of dietary protein modifies the response of the microsomal enzymes to the pharmacotoxicological activities of drugs and foreign compounds.

2.7.1 Hepatic Metabolism

Wood and Woodcock (1970, 1971) reported that force feeding a protein free diet to immature male rats for 5-7 days resulted in characteristic protein deficiency symptoms including weight loss, loss of hair and increased irritability when handled. Liver supernatant and microsomal UDPGT activity were significantly higher in animals fed a protein free diet compared to control animals on a 18% casein diet. They observed that UDPGT is a membrane bound enzyme and that its activity is dependant on the structural integrity of the membrane. Therefore, they suggested that increased UDPGT activity observed was due to alterations in endoplasmic reticulum phospholipid composition and/or the structure and not due to synthesis of new enzyme protein.

Graham et al (1974) investigated the effect of dietary protein on UDPGT activity and membrane phospholipid composition in male rats forced fed a protein free diet for 5-7 days or fed a 5% casein diet for 60 days. UDPGT activity in the liver microsomal fraction increased in both the acute and chronic protein deficiency states as compared to control animals fed a 18% casein diet. It appeared that protein deficiency altered the membrane phospholipid composition, as previously suggested by Wood and Woodcock

(1971). Microsomal membranes of the protein deficient animals contained significantly greater lysophosphotidylcholine and lysophosphatidylethanolamine than controls, 9-14% vs 0-2.7% of total phospholipids, respectively. Pretreatment with Phospholipase A and lysophosphatidylcholine increased the UDPGT activity in control preparations, leading Graham et al (1974) to conclude that the amount of microsomal membrane lysophosphatides in protein deficient animals caused the increased UDPGT activities of these preparations.

Hietanen (1980) investigated the effects of dietary protein on hepatic drug metabolism in 5 weeks old male rats fed a low (8%), normal (27%) or high (64%) protein diets for 4 weeks *ad libitum*. Following dietary treatment, PB (80 mg/kg BW) or 3-MC (100 mg/kg BW) were given for five days. They observed that hepatic C-P450 was unaffected by dietary treatments, while UDPGT activity was higher in animals fed the low protein diet. PB and 3-MC induced C-P450 regardless of diets, while UDPGT activity was the highest in the animals given a low protein diet. Since protein deficiency did not prevent microsomal enzyme induction, Hietanen (1980) confirmed that protein deficiency affected drug metabolism through changes in membrane structure and not through hampered protein synthesis (Graham et al, 1974).

Merrill and Bray (1982) questioned the physiological significance of the previous observations of Wood and Woodcock (1970, 1971), Graham et al (1974) and Hietanen (1980) because their conclusions were based solely upon *in vitro* experiments. Merrill and Bray (1982) fed weanling male rats either a low (7.5%), standard (15%) or high (45%) protein diets for 16 days and observed that *in vitro* C-P450 content increased with

increasing levels of dietary protein. These results were inconsistent with the observations of Hietanen (1980) but consistent with the observations of Clinton et al, (1977). *In vitro* hepatic UDPGT activity was significantly greater in rats fed a low protein diet as compared to rats fed 15 and 45% protein. Rats fed low dietary protein had a significantly shorter (37% decrease) chloral hydrate induced sleeping time than either the 15% or 45% protein groups. Thus, *in vitro* UDPGT enzyme activity correlated with the differences in chloral hydrate sleeping time confirming the physiological significance of the *in vitro* study. These results were not consistent with the literature. Previously it had been suggested that increased UDPGT activity in protein deficient animals was the result of proteins altering the phospholipid composition of the membrane (Wood and Woodcock (1971) and Graham et al (1974). However, Merrill and Bray (1982) studied fully activated enzymes, using detergents to eliminate any permeability barriers created by the membrane lysophosphatides, leading them to conclude that the enzyme effect observed may be due to increased enzyme levels.

Butler and Dauterman (1988) fed 8, 12 and 22% protein diets for 14 days to weanling male rats. Hepatic C-P450 content increased significantly with the level of dietary protein, while UDPGT activity was the greatest in animals on the low protein diet. They used hexobarbital induced sleeping time as a physiological bioassay of C-P450 activities. (C-P450 mediated enzyme activities correlate with hexobarbital induced sleeping time (Sipes and Gandolfi, 1991)). Butler and Dauterman (1988) observed decreased sleeping time with increased dietary protein confirming, again the physiological significance of the *in vitro* C-P450 activities. These results are consistent with previous

observations discussed in this review of literature.

Butler and Dauterman (1988) also studied the effect of dietary protein on bromobenzene hepatotoxicity by using pre and post treatment alanine aminotransferase activity levels as a measure of bromobenzene hepatotoxicity. While one-half of the 22% protein group died within the time course of the 8 hour assay, none of the animals in the 8 and 12% protein groups died during the experiment. The results suggested a protective effect of protein deprivation, with bromobenzene being more toxic at higher levels of dietary protein. This is consistent with the *in vitro* observations that demonstrate decreased C-P450 specific content and activity on low protein diets. Bromobenzene is biotransformed to reactive metabolites primarily through the action of C-P450 monooxygenases (Lau et al, 1984).

2.7.2 Effect of Age and Sex of Animal on Hepatic Phase I and Phase II Enzymes

Age related changes have the potential to alter xenobiotic disposition and toxicity of xenobiotics. Chengelis (1988) examined the effects of age and sex of the animal on hepatic phase I and phase II enzymes. Male and female rats were terminated at 4, 12, 26, 39, 51, 78, and 103 weeks of age. In both males and female rats, peak C-P450 activity was observed at 39 weeks of age, with a gradual decline thereafter. Males tended to have significantly greater C-P450 activities than females through 52 weeks of age. Peak C-P450 reductase activity in both males and females was observed at week 4, with no statistical difference between the sexes at this time. After week 4 activity declined, with females having greater enzyme activity than males leading to significant differences

at subsequent time points. The investigators suggested that the activity of the microsomal MFO were lower in rats over 39 weeks of age and especially in those over 78 weeks of age.

UDPGT activity in males and females towards p-nitrophenol ranged from 480-1050 nmol/min per g liver, with maximal activity observed at 39 weeks of age. While male rats had significantly greater activity between weeks 12-39, no differences were seen in the females over the study period. Activity with chloramphenicol as a substrate ranged from 3.8 to 10.3 nmol/min per g liver with no consistent age or sex relationship. Differences between sexes for UDPGT activity were largely quantitative, that is both sexes had similar age related patterns, but males tended to have greater activity. The investigators suggest that total enzymic capability of UDPGT was greatest in animals from 26-78 weeks of age. On the other hand C-P450 activity during a similar period (following 39 weeks) tended to reach minimal levels.

2.7.3 Extrahepatic Metabolism

Limited information is available on the effect of dietary protein on extrahepatic C-P450 and UDPGT activity. Catania and Carrillo (1990) studied the effects of protein diets on liver and intestinal UDPGT activity in weanling male rats fed a standard 24% casein diet (ND) or a 6% casein low protein diet (LPD) for 7 days. One of the LPD treatment group was sacrificed after 7 days, while a second treatment group was refed the ND and sacrificed after different intervals of refeeding. After 7 days of LPD feeding liver UDPGT increased by 58%, whereas intestinal UDPGT increased by 178%, a three

fold greater response in the intestine than in the liver. Enzyme activity returned to control values after 2 days of refeeding with ND. Catania and Carrillo (1990) suggested that increased enzyme activity is an adaptive response to unfavourable conditions. This intestinal detoxification system enhancement demonstrates the potential of intestinal tissue to metabolize xenobiotics.

Tutelyan et al (1990) investigated the effects of dietary protein on xenobiotic metabolizing enzyme activity in the liver and small intestine of male rats fed low (5%), control (18%) and high (32%) protein for two months. Liver C-P450 was moderately decreased in rats fed the low protein diet. Liver GT_1 activity was higher in the animals fed the low protein diet, while GT_2 markedly decreased in animals fed the low protein diet. They demonstrated that both decreases and increases in the dietary protein level were accompanied by changes in the activity of enzymes involved in detoxification. In contrast to the liver, intestinal GT_1 and GT_2 activities were the highest on the low protein diet. They suggested dietary factors may differently affect drug metabolism in various tissues, and it is possible that each tissue contains a unique complement of detoxifying enzymes, each with a unique pattern and distribution.

The results of Catania and Carrillo (1990) and Tutelyan et al (1990) demonstrate intestinal adaptation to dietary modulations by proteins. In addition Tutelyan et al (1990) demonstrated that the phase II isoenzymes respond to dietary modulations, that there was intra and inter organ variation; adaptive responses which may have toxicological and pharmacological consequences.

Swann and McLean (1971) investigated the effects of dietary protein on the metabolism of dimethylnitrosamine (a selective kidney toxin) in rats by feeding animals a protein free high carbohydrate diet for 7 days. Protein free diets impaired dimethylnitrosamine metabolism in liver slices as measured polarographically. However, protein free diets in kidney slices did not impair dimethylnitrosamine metabolism. Swann and McLean (1971) suggested that impaired hepatic dimethylnitrosamine metabolism was due to changes in enzyme activity that resulted from protein deficiency. To support this notion, that protein played a more positive role than carbohydrate in toxicity by dimethylnitrosamine, they relied upon previous work in which CCl_4 toxicity was reversed by the addition of only 10% casein to a protein free diet (McLean and Mclean, 1966),

Swann et al (1980) investigated the effect of dietary protein on kidney tumour induction using a single dimethylnitrosamine dose. Young male rats, 5-6 weeks old, were acclimatized on chow diet for 5-7 days, randomly assigned to a control group, fed chow or a semi synthetic protein free diet for 7 days and then given 40 mg/kg BW of dimethylnitrosamine. The 24 week mortality rate were similar, 27% and 24% for the control and protein groups respectively, however, after 104 weeks the mortality rates were significantly different ($p < 0.01$) with 53% and 76% for the control and treatment groups respectively during this period. Clearly, protein free diet increased the susceptibility of the animals to the action of the renal carcinogen. Swann et al (1980) suggested that pretreatment with the protein free diet alters nitrosamine pharmacokinetics so that a greater proportion of any dose is activated to the carcinogen in the kidney.

2.7.4 Importance of Hepatic versus Extrahepatic First Pass Metabolism

First pass metabolism is defined as the reduced systemic availability of drugs following oral administration, even in the presence of complete absorption. The two processes responsible for this effect are gastrointestinal and hepatic extraction and/or metabolism. Although both the liver and the gut contribute to first pass metabolism, it is important to know the relative contribution of either the liver or gut in producing this first pass effect.

Iwamoto et al (1982) investigated hepatic and gastrointestinal first-pass metabolism of aspirin in male rats. A pharmocokinetic model that differentiates presystemic (preabsorptive, gut epithelial and hepatic) first pass metabolism was used (Colburn, 1979). The first-pass effect of aspirin was measured by comparing the plasma concentration after intravenous, oral or intraportal administration (10 mg/kg BW) of the drug. Within 48 hours of oral administration approximately 86% of the dose was excreted in the urine as salicylic acid and its glucuronides and sulphate conjugates, suggesting that gastrointestinal absorption of aspirin was almost complete in rats. Following systemic and intraportal administration the cumulative biliary excretion of total salicylates (aspirin + salicylic acid + conjugates was less than 0.25% and 0.22% of the dose respectively. At 120 minutes post infusion, biliary excretion of total salicylates was determined to be less than 0.3% of the dose for both routes. These results demonstrated that orally administered aspirin was subject to first-pass metabolism in both the liver and gut and that the gastrointestinal first-pass effect was more important than the hepatic effect.

Cassidy and Houston (1984) investigated the *in vivo* capacities of the intestinal mucosa, liver and lung to conjugate phenol (0.4-15.0 mg/kg BW) in male rats. At low doses (0.4 mg/kg BW), the ability of intestinal and hepatic enzymes to conjugate phenol was comparable. However, as the dose of phenol increased the capacity of the liver was rapidly exceeded, whereas the intestine maintained efficient conjugation over a wide dose range. They suggested that the high affinity, high capacity intestinal enzyme systems would reduce the amount of dietary phenols reaching the circulatory system, thereby reducing the toxic load on other detoxification systems. These results were consistent with Iwamoto et al (1982).

Iwamoto et al (1987) determined the effects of age on gastrointestinal and hepatic first pass metabolism of levadopa in 5-104 week old male rats. The gastrointestinal and hepatic contribution to the overall first pass metabolism was estimated separately after the drug was administered intravenously, orally and intraportally (20 mg/kg BW). The contribution of the gut to the overall first pass metabolism of levadopa was greater than that of the liver for all age groups. In the gut the overall and intestinal first pass metabolism of levodopa was the greatest in 11-week old rats, while in both young (5-7 weeks) and older (52-104 weeks) rats the metabolism was relatively low. In contrast to the gut, hepatic first pass metabolism did not show any significant age dependant change. The investigators suggested that intestinal first pass metabolism may be a determinant in the overall first pass metabolism of levadopa.

2.8 SUMMARY

Xenobiotics and endogenous substances such as fatty acids, vitamins and hormones are metabolized by the drug metabolizing enzyme system. This metabolism is carried out by phase I and phase II enzymes located in the cell cytosol and endoplasmic reticulum. The enzymes metabolize these substances via oxidative, reductive and conjugative pathways making them more water soluble thereby facilitating their excretion from the body.

Dietary proteins can alter the rate of metabolism of xenobiotics via induction of phase I or phase II enzymes. For example, UDPGT activity is higher in animals given a protein free or low protein diet (0-8%) as compared to controls (15-27%) and high protein diets (32-64%). There is conflicting evidence on the effect of protein diets on C-P450 activity. The observed differences could be due to the differences in the ages of the animals used in the various studies. The modulating effects of low protein diets have been related to the alteration in the phospholipid component of the membrane.

Research suggests that protein deficiency increases vulnerability to toxicological insults. Since the liver is the primary drug metabolizing tissue, the interaction between dietary protein and the toxic expression of xenobiotics has been examined in hepatic drug metabolizing systems. However, detoxifying enzyme systems are not confined to the liver and phase I and phase II systems have been demonstrated in the lungs, kidneys and small intestine. Although extrahepatic systems have been documented the physiological significance and control mechanisms are not well understood. Yang and Yoo (1988) in a systematic review of the subject suggest that dietary effects on drug metabolism by

extrahepatic tissues are largely unknown and remain to be studied. The objective of this study therefore, was to examine the effect of dietary modulations of high and low levels of protein on hepatic and extrahepatic tissue.

Chapter III

MATERIALS AND METHODS

3.1 Animals

Male weanling Sprague-Dawley rats (50-60 g) were purchased from the University of Manitoba central animal care facility. They were housed separately in stainless steel cages and fed *ad libitum* the standard 15% protein diet for an adaptive period of 5 days. Animals were then switched to 7.5% low, 15% standard, or 45% high protein diets and fed ad libitum for 7 or 14 days. The animals were weighed and fed every second day.

3.2 Diet Formulation

Diets were formulated according to the National Research Council's guidelines set forth in the Nutrient Requirements of Laboratory Animals (1978). The level of corn oil in the diet was maintained at 5% in accordance with the recommendation of the American Institute of Nutrition (1977) to ensure that the diet was sufficient in essential fatty acids. Specifics of the diet formulation are provided in Table 1. The diet used followed the Merrill and Bray (1982) study.

TABLE 1

Diet Composition (% Weight)

Ingredient	Low Protein (7.5%)	Standard Protein (15%)	High Protein (45%)
Calories	410	410	410
Casein	8.62	17.24	51.72
Corn Starch	35.76	31.38	13.84
Glucose	35.76	31.38	13.84
DL-Methionine	0.15	0.30	0.90
Mineral Mix	3.50	3.50	3.50
Vitamin Mix	1.00	1.00	1.00
Choline Bitartrate	0.20	0.20	0.20
Corn Oil	5.00	5.00	5.00
Lard	5.00	5.00	5.00
Fibre	5.00	5.00	5.00

3.3 Experimental Design

Table 2 summarizes the experimental design. Forty-eight animals were fed a standard protein diet for a 5 day adaptive period. Rats were randomly assigned and fed *ad libitum* the low, standard, or high protein diet for 7 or 14 days. Animals were killed at the end of 7 or 14 days of experimental diets.

Table 2

Experimental Population (48)

Parameter	# of Animals	Days on Diets	Animals On Low Protein Diets	Animals on Standard Protein Diets	Animals On High Protein Diets
Adaptive period of study	48 Animals	5		48	
Day 7 Termination	24 Terminated	7	8	8	8
Day 14 Termination	24 Terminated	14	8	8	8

3.4 Experimental Protocol

3.4.1 Animals

Animals were killed by decapitation and their intestine, liver and kidneys were excised and cleared of surrounding fat and mesentery. The kidneys and liver were immediately frozen in liquid nitrogen and kept frozen at -80°C until required for microsome preparation. Intestinal microsomes were prepared on the day of termination and stored at -80°C until required for enzyme assay.

3.4.2 Microsome preparation

Microsomes were prepared from the intestine (Stohs et al, 1976), kidney (Jakobsson, 1974) and liver (Hassen, 1993). Microsomal protein was determined using the Lowry et al assay (1951).

3.4.2.1 Intestinal Microsome Preparation

The intestine was exposed and the distal and proximal ends identified and sutured. The intestine was flushed several times with ice cold 0.05 M Tris HCL buffer in isotonic KCL (pH 7.8). The cleaned intestine was placed in ice cold saline solution on ice before processing. The intestine was placed on a ECP-150 Electronic cold plate and cut, slit open and the mucosa scraped from a 20 cm section with a glass slide onto a weighing boat on ice. This process was repeated with three intestine sections to obtain mucosa from a total of 60 cm of the intestine. The mucosa was weighed and suspended in 20 ml

0.05 M Tris HCL buffer in homogenizing tubes. Trypsin inhibitor (5 mg/g weight of mucosa), glycerol (20% v/v final concentration) and heparin 3 U/ml were added to this suspension. The homogenizing tubes were maintained in ice throughout the process of microsome preparation.

The suspended mucosa was homogenized in a Polytron homogenizer for 60 s and the homogenate centrifuged at 10,000 g for 20 min. Following centrifugation the post mitochondrial supernatant was transferred to clean chilled centrifuge tubes and centrifuged at 105,000 g for 60 min in a Beckman L5-50B ultracentrifuge. The supernatant was discarded and the microsomal pellet suspended in 0.05 M Tris-HCL buffer (1 ml/g mucosa) and homogenized for 15-20 s to insure that all microsomes in the tube were dissolved in the suspending medium. The microsomal protein was determined by the Lowry method (1951) using a 1/100 dilution with distilled water. The microsome was frozen at -80°C.

3.4.2.2 Kidney Microsome Preparation

The kidneys were excised, decapsulated and placed in ice cold 0.25 M sucrose. Kidneys were perfused *in situ* with ice cold saline as required for C-P450 c reductase determination. The kidneys were sagitally sectioned, medulla and papillae removed, minced and 2 g of the tissue suspended in 7.5 ml of 3 mM Tris HCL buffer pH 7.4 and homogenized in a Potter-Elvejhem Homogenizer for 90 s using a chilled teflon pestle. The homogenizing tubes were maintained on ice throughout the process of microsome preparation. Following homogenization the sucrose concentration was adjusted to 0.25

M by adding 2.5 ml of 1.0 M sucrose. The homogenate was centrifuged at 10,000 g for 20 min to remove cell debris, nuclei, mitochondria and lysosomes in a Beckman L5-50B ultracentrifuge. The supernatant was transferred to clean, chilled centrifuge tubes and recentrifuged for 60 min at 105,000 g. The resulting supernatant was discarded and the microsomal pellet was suspended in 4 ml 0.25 M sucrose (1 ml/0.5 g tissue) and homogenized for 15-20 s to insure that all microsomes were dissolved in the suspending medium. The microsomal protein content was determined by the Lowry method (1951) using a 1/200 dilution with distilled water. The microsome was frozen at -80°C.

3.4.2.3 Liver Microsome Preparation

Two different buffer solutions were used to prepare liver microsomes. KCL in 0.25 M buffer was used to prepare microsomes for UDPGT assay while a sucrose Tris buffer was used to prepare microsomes for C-P450 c reductase assay. Frozen liver was thawed, cut into small pieces and 2.0 g weighed for each preparation. One of the 2.0 g portions was suspended in a chilled homogenizing tubes containing 4 ml of ice-cold KCL in 0.25 sucrose solution, while the other 2.0 g portion was suspended in 4 ml sucrose tris buffer.

The homogenizing tubes were maintained on ice throughout the process of microsome preparation. The liver was homogenized in a Potter-Elvejhem Homogenizer for 90 s using a chilled teflon pestle. The homogenate was transferred with a pasteur pipette to an ultracentrifuge tube and centrifuged for 20 min at 10,000 g in a Beckman L5-50B ultracentrifuge. The resulting postmitochondrial fraction was transferred to

chilled centrifuge tubes and recentrifuged for 60 min at 105,000 g. The supernatant was removed and discarded leaving the microsomal pellet.

An additional microsomal wash was carried out with each of the two microsomal pellet preparations. The pellets were homogenized for 10-15 s with 2.0 ml cold KCL in sucrose solution and sucrose tris buffer, respectively. The homogenate was centrifuged at 105,000 g for 45 min and the supernatant was discarded, the microsomal pellets resuspended in 2.0 ml (1 ml/g liver) cold KCL in sucrose solution and sucrose tris buffer, respectively. The microsomal pellets were rehomogenized for 15-20 s to insure that all microsomes were dissolved. The microsomal protein content was determined by the Lowry et al method (1951) by using a 1/400 dilution with distilled water. The remaining microsome was frozen at -80°C.

3.4.3 Protein Estimation

The method of Lowry et al (1951) was followed. Stock bovine serum albumin (BSA) 1 mg/ml was used to prepare the working standard. A BSA standard curve was run initially to verify that the line was straight and intersected zero. Absorbance readings of the standard and samples were taken against distilled water at 700 nm in the SP6-300 spectrophotometer.

Calculation :

Protein mg/ml =

Concentration of sample(X) x Absorbance of standard = Concentration of standard x Absorbance of sample

3.4.4 Enzyme Assays

UDP-Glucuronyltransferase (GT_1 and GT_2) (Mulder and Van Doorn, 1975) and cytochrome P-450 c reductase (Jeffrey et al, 1977) were assayed in the intestine, kidney and the liver microsomes.

3.4.4.1 Cytochrome P-450 c Reductase Assay

The method of Jeffrey et al (1977) was followed. Validation of the method was carried out for the intestine, kidney and liver microsomes to determine that the enzyme system was saturated, and the protein concentration was appropriate (Appendices C, D and E). It was concluded that the method was working under optimum conditions and no modifications were necessary. Liver microsomes were diluted to 1.0 mg/ml protein, kidney and intestinal microsomes were diluted to 0.8 mg/ml for the C-P450 c reductase assay.

Principle:

NADPH + oxidized Cytochrome C ----------▸ NADP + reduced cytochrome C

One unit of enzyme reduced one nanomole of cytochrome-C / min, at 37°C and pH 7.4, in the presence of NADPH. The reaction was monitored via the increase in absorbance at 550 nm (reduced cytochrome-C).

Reagents:

Homogenizing buffer: 0.25M Sucrose/5mM Tris, pH 7.4.

Working buffer: 55.5 mM Tris/167 mM KCl/11.1mM MgCl2, Ph 7.4.

Cytochrome-C substrate solution: 222 μM cytochrome-C in working buffer prepared fresh daily.

40 mM NADPH: Prepared frequently and stored frozen in daily aliquots.

Assay:

Microsomes were diluted to the required protein dilution with Tris buffer. Cytochrome-C substrate solution was prepared and 1.8 ml pipetted into each of three 4.5 ml disposable chilled cuvets. The diluted microsome was vortexed and 100 μl of the microsome pipetted into each of 3 the prepared cuvets. The cuvets were placed in a 37°C waterbath, mixed and incubated for 2 minutes. Once incubated the cuvets were placed in the spectrophotometer. A 100 μl of distilled water was added to the blank, while a 100 μl of 40 mM NADPH was added to the sample and both were stirred. A single blank and duplicate samples were run for each animal and the change in absorbance was read at 550 nm over 4.5 minutes.

Determination of Activity:

The Specific Activity (SA) was calculated using the following calculation.

$$SA\ (U) = \frac{A/\ min \times 2\ ml(reaction\ volume) \times 1000}{\text{Molar absorbance coeff. reduced cyt. c (22.6)} \times \text{mg protein in cuvette}}$$

The Rate Analysis Program on the Milton Roy Spectronic 3000 spectrophotometer was used to confirm the calculated Specific Activity.

3.4.4.2 UDP-Glucuronyltransferase Assay

Method Development:

The method of Mulder and Van Doorn (1975) for assaying UDPGT enzyme activity was optimized for the intestine, kidney and liver. α-naphthol was used as a substrate for the GT_1 assay and p-hydroxy biphenyl was used as a substrate for GT_2 assay. Optimization involved running blanks and samples in duplicates for each tissue at different protein (0.5, 0.8 and 1 mg/ml) and triton concentrations.

The initial intestinal and kidney assays did not work. There was little difference between the blank and sample readings for the GT_1 or GT_2 assay at different triton and protein concentrations. Problems with the blank solution were thought to be the reason for the high background readings in the intestine and kidney. As an alternative, a boiled blank was used to assay GT_1 and GT_2 in the intestine and kidney. Although a boiled blank gave lower blank readings as compared to the sample, there was little change in the sample over time. The linked assay for UDPGT enzyme requires an optimum pH of 7.3. On testing, it was determined that the pH of the assay solution dropped to between

3.5-4.0 on addition of the GT_1 and GT_2 substrates. On adjusting the pH to 7.3 with sodium hydroxide the sample showed a change in activity over time. GT_1 and GT_2 enzyme assays in the intestine and kidney were then optimized for triton and protein concentrations (Appendices F, G, H, I).

In order to maintain consistency among tissues a boiled blank and an adjustment of the pH to 7.3 was also followed for liver GT_1 and GT_2 enzyme assay (Appendices, J and K).

Principle:

(1) R-OH + UDP-glucuronate ----------▸ R-O-glucuronide + UDP

(2) UDP + phosphoenolpyruvate ----------▸ UTP + pyruvate

(3) Pyruvate + NADH + H ----------▸ lactate + NAD

One unit of UDPGT enzyme conjugates 1 nmole of UDP-glucuronate/hour, at 30°C and pH 7.3. The assay is based on measurement of UDP production during the glucuronidation reaction (1), which is linked by reactions (2) and (3) to the conversion of NADH into NAD. This conversion was monitored spectrophotometrically via changes in absorbance at 340 nm. Glucuronidation of any substrate (R-OH) which does not affect the coupling reactions can be measured.

Reagents:

a) "Acceptor" Substrates:

α-naphthol was used as a substrate for the GT_1 assay and p-hydroxy biphenyl was used as a substrate for the GT_2 assay. Both were dissolved in ethanol-water, 1:1, at a concentration of 4mM. Substrate and blank solutions were prepared fresh daily.

b) Blank Solution:

The amount of blank solution needed daily was calculated by multiplying the number of samples X 6 ml, then adding 4 ml extra.The blank solution contained, per 100 ml:

Reagent	Volume
0.105M Tris buffer,pH 7.3	95 ml
MgCl2	0.1352 g
Phosphoenolpyruvate	0.0056 g
NADH	0.0210 g
Pyruvate kinase (540 U/mg)	0.0012 g
Lactate dehydrogenase (5000 U/ml)	0.017 ml
Acceptor substrate (4 mM in EtOH-H2O,1:1)	5.0 ml
D-Saccharolactone (Intestine 10mM conc)	0.2561 g

UDP Glucuronic acid (ammonium salt), 0.1346 g/100ml was added to half of the prepared "blank solution" to prepare the substrate.

Assay

The assay was performed in chilled 4.5 ml disposable cuvettes in a Milton Roy Spectronic 3000 spectrophotometer. Liver, kidney and intestinal microsomes were diluted to 1.0 mg protein/ml concentration with triton in 0.154M KCl for GT_1 and GT_2 assays. Since boiled blanks were used, microsomal dilutions for the blank and sample were done in separate vials. Blank and samples were run in duplicates.

The blank and sample cuvettes were pre-incubated in a waterbath at 37°C for 5 minutes. While the cuvettes incubated, the vial containing the blank microsomal dilution was boiled. Once incubated the cuvettes were placed in the spectrophotometer and 0.5 ml of the diluted blank or sample microsome were added to the blank and sample cuvette respectively. Absorbance readings for the blanks and samples were taken over five minutes at 340 nm using distilled water as a reference.

Determination of Activity:

The rate of change in absorbance for the duplicate assays of the blank and sample were averaged for the duration of the linearity of the reaction. The averaged values of the blank were subtracted from the average value of the sample. The enzyme activity or Specific Activity (SA) was calculated as follows:

$$SA = \frac{A/\text{minute} \times 1000 \times 2 \text{ ml (reaction vol)}}{\text{mg protein in cuvette} \times \text{molar absorption coeff. of NADH (6.22)}}$$

The specific activity represents the conversion of X nanomoles of NADH to NAD /minute/mg protein, which is equivalent to the conjugation of X nanomoles of UDP-GA (by UDP-GT)/minute/mg protein, or, in short,"units of UDPGT activity".

The Rate Analysis Program on the Milton Roy Spectronic 3000 Spectrophotometer was used to confirm the calculated Specific Activity.

3.5 Statistical Analysis

Data was analyzed using the Statistical Analysis System (SAS), version 6.06. Since the three treatments (diets) were evaluated at different time points (day 7 and 14) a repeated measures analysis of variance (Anova) was used to determine the effect of diet and day (time) on the enzymes. To determine differences between dietary treatments and within diets at the two time points, multiple comparisons using paired t tests were used.

Chapter IV

RESULTS

4.1 Animal Weight

Food intake and animal weight gain were affected by diet. During both feeding periods food intake on the high protein diets was significantly lower than that observed in groups eating the standard and low protein diets (Table 3). Animals on the standard protein diet had the highest weight gain, while rats on the high protein diets had the lowest weight gain (Table 4; Figure 5). Difference in weight gain reflected the reduced food intake in high protein diet animals compared to the low and standard groups.

4.2 Enzyme Assays

The effects of protein diets on UDPGT and C-P450 c reductase activities can be analyzed in terms of Diet effect and Day effect. For the Day effect, GT and reductase activities were examined over two time periods within dietary groups. In this study, the effects of diet on reductase and GT_1 and GT_2 activity were examined at time points 7 and 14 days, while in other studies (Hietanen, 1980; Catania and Carrillo, 1990) the effects after a single time period were investigated.

Table 3: Effect of Dietary Protein on Food Intake (g)

Variable	n	Food Intake	Low Protein Diet	Standard Protein Diet	High Protein Diet
Adaptive Period					
Day 3	48	11.2 ±1.2			
Treatment (Day 7)					
Day 6	16	-	14 ± 1.9[a]	14 ± 1.1[a]	13 ± 1.5[a]
Day 8	16	-	16 ± 1.4[a]	15 ± 2.1[a]	11 ± 1.7[b]
Day 10	16	-	18 ± 2.2[a]	16 ± 1.3[b]	12 ± 1.8[c]
Day 13	16	-	19 ± 2.2[a]	17 ± 1.6a[a]	12 ± 2.3[b]
Treatment (Day 14)					
Day 15	8	-	21 ± 2.1[a]	21 ± 1.3[a]	14 ± 1.4[b]
Day 17	8	-	21 ± 1.4[a]	19 ± 2.1[a]	13 ± 1.8[b]
Day 20	8	-	23 ± 3[a]	20 ± 2.2[b]	11 ± 2.3[c]

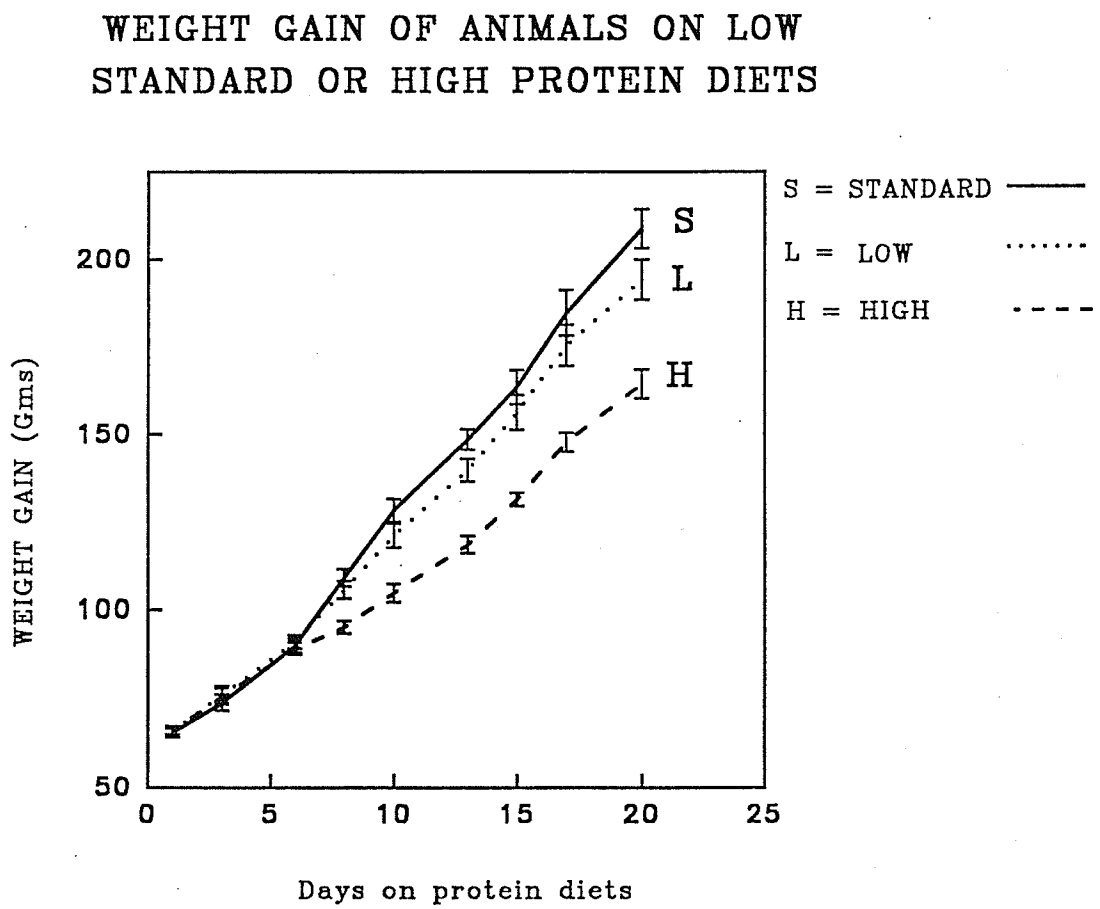
Means ± SD denoted with different letter superscripts on each day are significantly different ($p < 0.01$).

Table 4: Effect of Dietary Protein on Body Weight (g)

Variable	n	Body Weight	Low Protein Diet	Standard Protein Diet	High Protein Diet
Adaptive Period					
Day 1	48	66 ± 4.3			
Day 3	48	75 ± 9.3			
Treatment (Day 7)					
Day 6	16	-	91 ± 7[a]	90 ± 8[a]	89 ± 7[a]
Day 8	16	-	106 ± 10[a]	109 ± 10[a]	95 ± 7[b]
Day 10	16	-	121 ± 14[a]	129 ± 13[a]	105 ± 10[b]
Day 13	16	-	140 ± 13[a]	148 ± 12[a]	118 ± 10[b]
Treatment (Day 14)					
Day 15	8	-	156 ± 14[a]	163 ± 14[a]	131 ± 5[b]
Day 17	8	-	175 ± 17[a]	185 ± 18[a]	148 ± 8[b]
Day 20	8	-	194 ± 17[a]	209 ± 16[a]	164 ± 12[b]

Means ± SD denoted with different letter superscripts on each day are significantly different ($p < 0.01$).

Figure 5


WEIGHT GAIN OF ANIMALS ON LOW
STANDARD OR HIGH PROTEIN DIETS
S = STANDARD
L = LOW
H = HIGH
S
L
H
WEIGHT GAIN (Gms)
200
150
100
50
0
5
10
15
20
25
Days on protein diets

4.2.1 Cytochrome P-450 c Reductase

Following 7 days of dietary treatment, intestinal reductase activity was the greatest on the high protein diet with significant differences observed between the low and the high treatment groups (110-136 nmol/min/mg protein, respectively, $p < 0.01$) as well as between the standard and the high treatment groups (111-136 nmol/min/mg protein, respectively, $p < 0.01$) (Table 5; Figure 6). Significant differences were not observed between the low and standard protein groups. Following 14 days of dietary treatment, significant differences between the low, standard and high protein diets (110-90-135 nmol/min/mg protein, respectively, $p < 0.01$) were observed (Table 5; Figure 6).

No significant differences between day 7 and 14 were observed for intestinal reductase activity within the low and high dietary protein groups (Table 5; Figure 7). However, for standard protein, reductase activity was significantly lower after 14 days (90-111 nmol/min/mg protein, respectively, $p < 0.01$) of treatment compared to 7 days respectively (Table 5; Figure 7).

Following 7 days of dietary treatment kidney reductase activity was the greatest on the high protein diet with significant differences observed between the low and the high treatment groups (55-73 nmol/min/mg protein, respectively, $p < 0.01$) as well as between the standard and the high treatment groups (58-73 nmol/min/mg protein, respectively, $p < 0.01$) (Table 5; Figure 8).

Table 5: Effect of Dietary Protein on Reductase Activity: Diet and Day Effect.

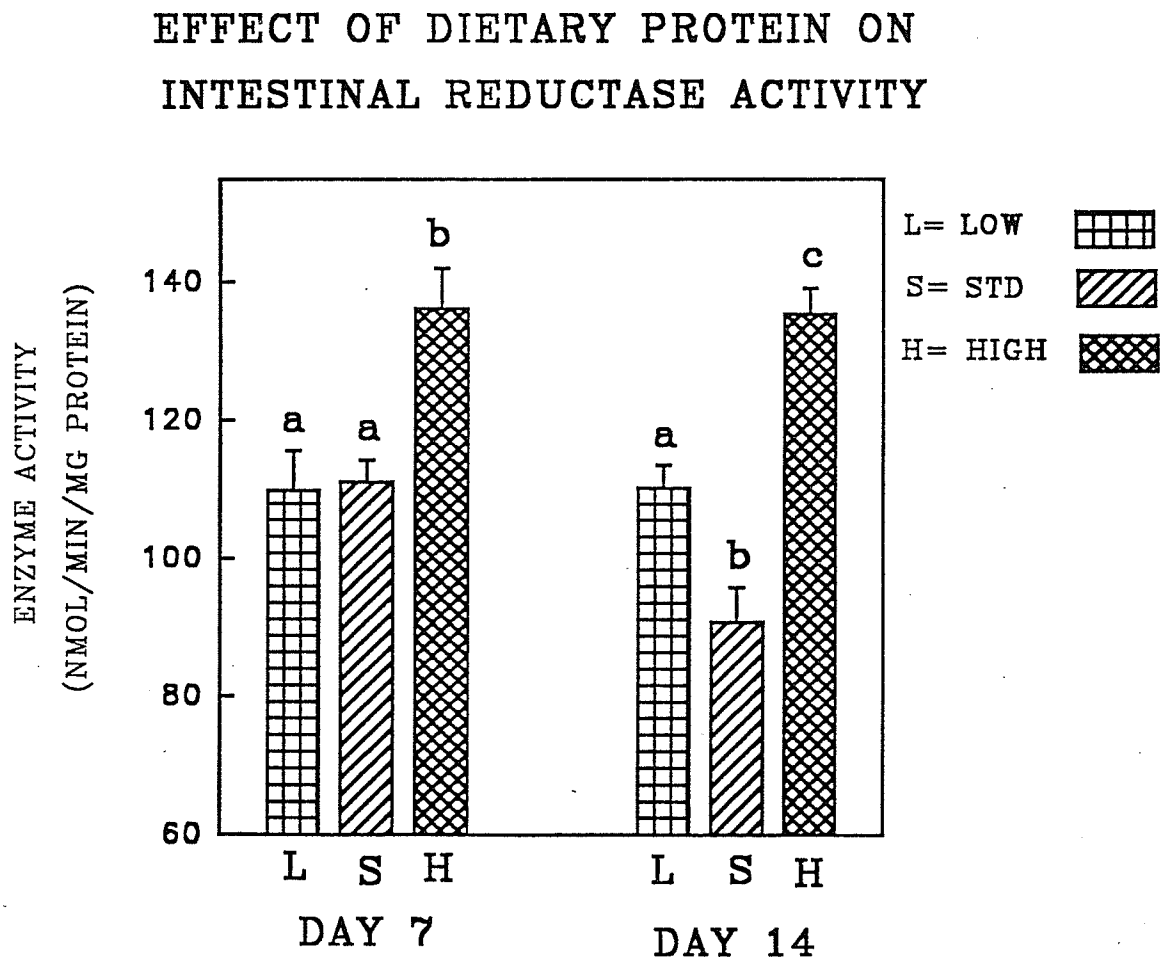
Tissue	Day	n	Low	Standard	High
Intestine	7	8	$110 \pm 16^{a}_{I}$	$111 \pm 9^{a}_{I}$	$136 \pm 17^{b}_{I}$
	14	8	$110 \pm 9^{a}_{I}$	$90 \pm 14^{b}_{II}$	$135 \pm 11^{c}_{I}$
Kidney	7	8	$55 \pm 9^{a}_{I}$	$58 \pm 6^{a}_{I}$	$73 \pm 13^{b}_{I}$
	14	8	$54 \pm 7^{a}_{I}$	$57 \pm 4^{a}_{I}$	$72 \pm 8^{b}_{I}$
Liver	7	8	$68 \pm 8^{a}_{I}$	$84 \pm 7^{b}_{I}$	$110 \pm 9^{c}_{I}$
	14	8	$70 \pm 8^{a}_{I}$	$92 \pm 11^{b}_{I}$	$115 \pm 7^{c}_{I}$

Diet effect and Day effect: Each value is expressed in nmol/min/mg protein and represents means $\pm$ SD.

Diet effect: Different letter superscripts for each tissue on each, day 7 and day 14, significantly different ($p < 0.01$).

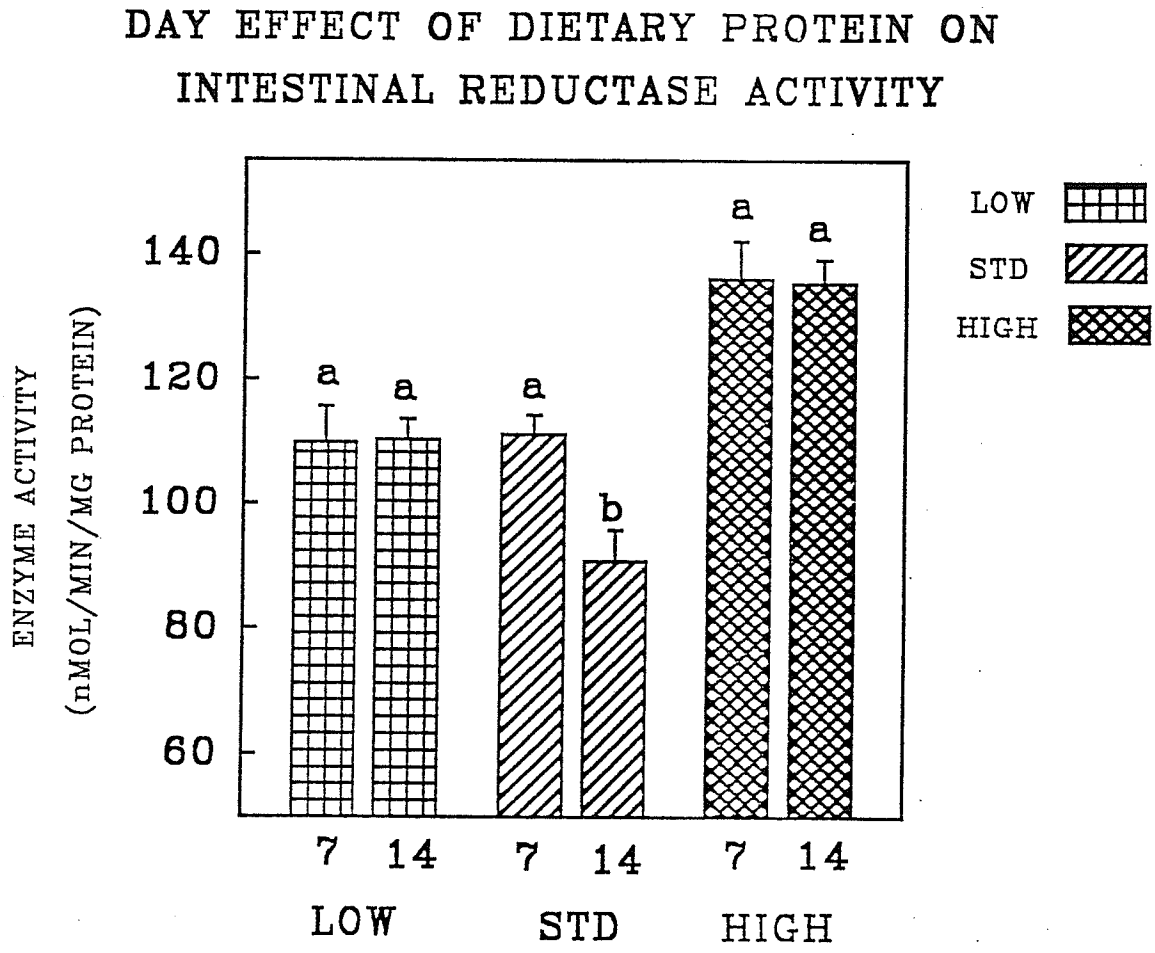
Day effect: II (subscripts) significantly different from day 7 ($p < 0.01$).

Figure 6



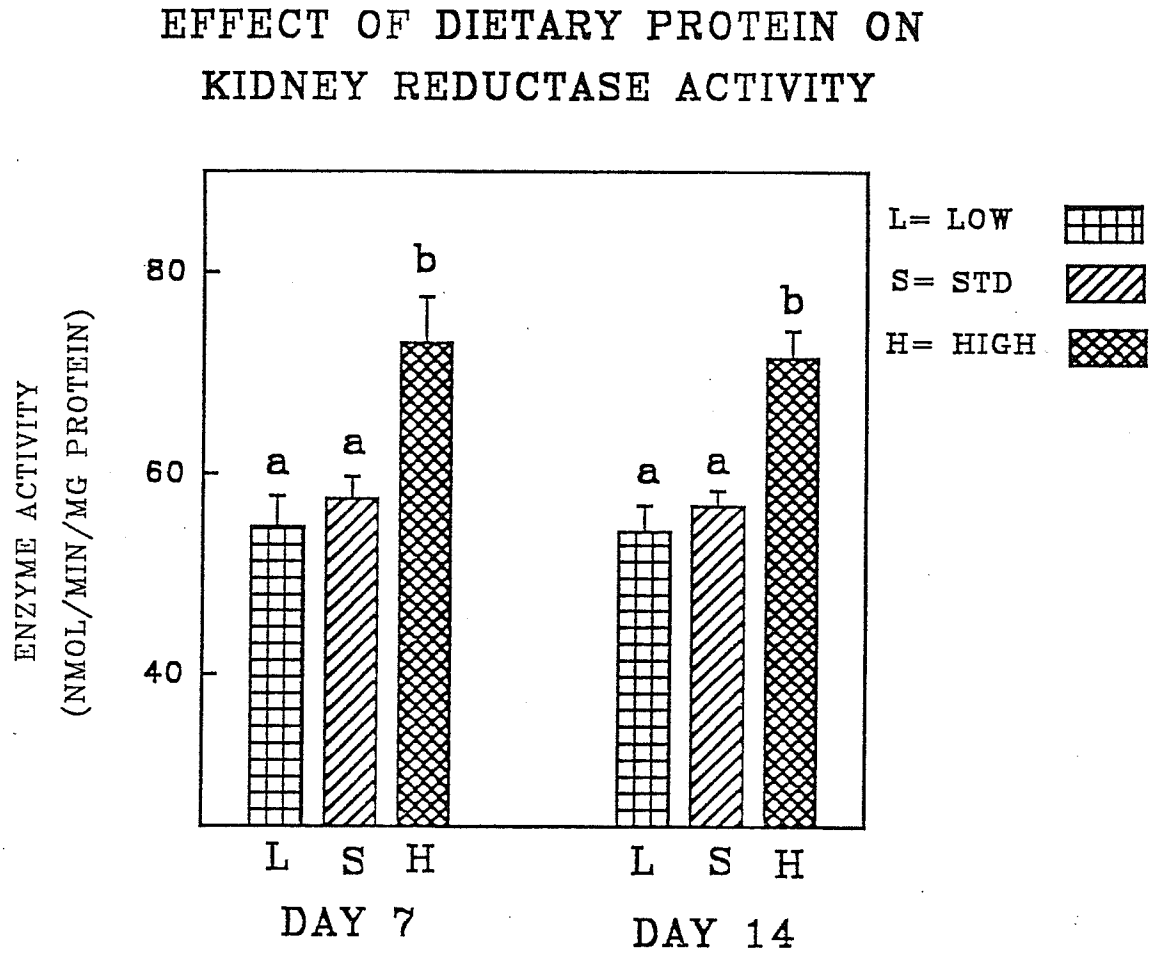
Means ± SEM (n=8) denoted with different letters on each day are significantly different ($p < 0.01$)

Figure 7



Means ± SEM (n=8) denoted with different letters within each dietary group are significantly different ($p < 0.01$)

Figure 8



Means ± SEM (n=8) denoted with different letters on each day are significantly different ($p < 0.01$)

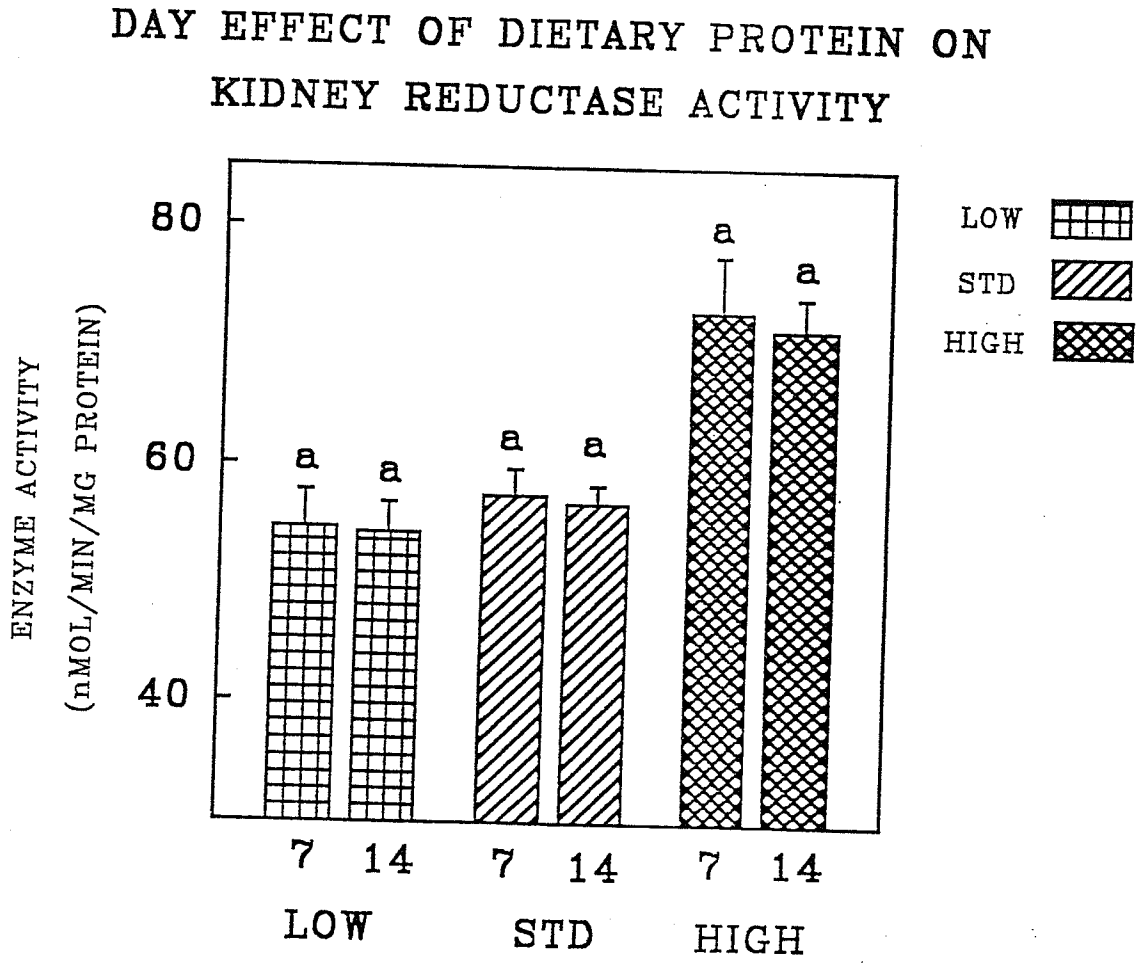
Following 14 days of dietary treatment, kidney reductase activity was the greatest on the high protein diet with significant differences observed between the low and the high treatment groups (54-72 nmol/min/mg protein, respectively, $p < 0.01$) as well as between the standard and the high treatment groups (57-72 nmol/min/mg protein, respectively, $p < 0.01$) (Table 5; Figure 8). On both treatment days no significant differences were observed between the low and standard treatment groups (Table 5; Figure 8).

No significant differences between day 7 and 14 were observed for kidney reductase activity within any dietary treatment (Table 5; Figure 9).

Following 7 days of dietary treatment, animals on high protein diets had significantly greater hepatic reductase enzyme activity than those on a standard protein diet, than those on a low protein diet (68-84-110 nmol/min/mg protein, respectively, $p < 0.01$) (Table 5; Figure 10). Fourteen days of dietary treatment did not alter this enzyme profile, with animals on the high protein diet having significantly greater hepatic reductase enzyme activity than those on a standard protein diet, than those on a low protein diet (115-92-70 nmol/min/mg protein, respectively, $p < 0.01$) (Table 5; Figure 10).

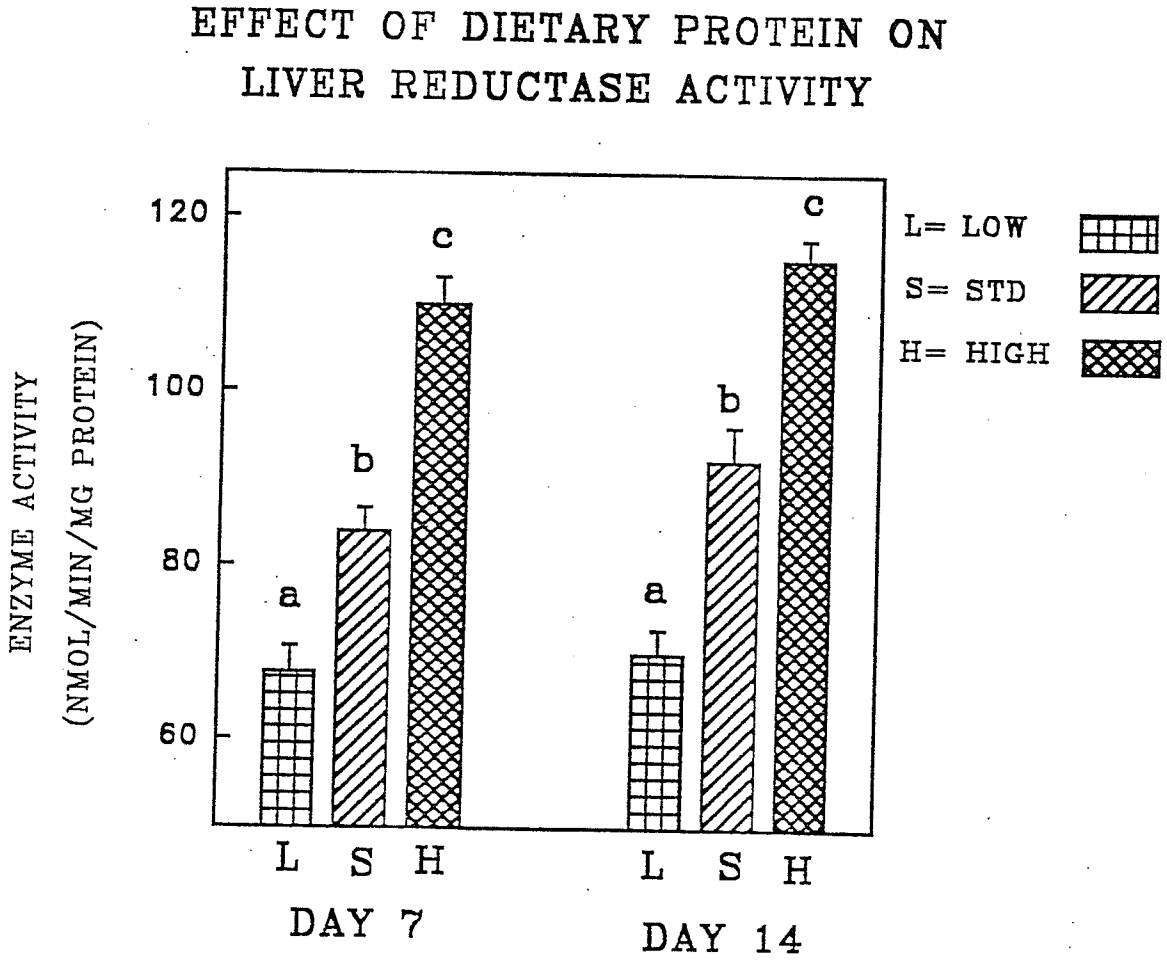
No significant differences between day 7 and 14 were observed for hepatic reductase activity within any dietary treatment (Table 5; Figure 11).

Figure 9



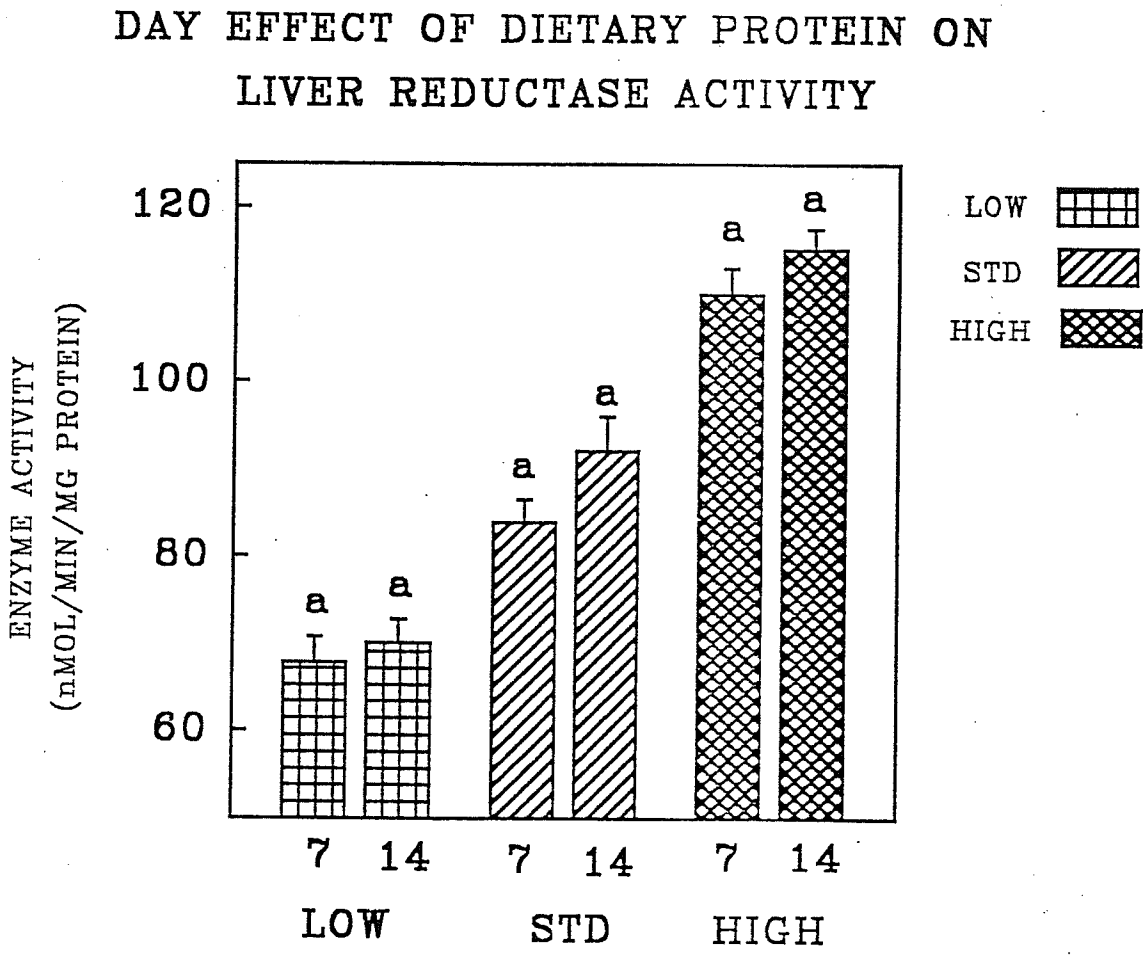
No significant difference between means ± SEM (n=8) within each dietary group

Figure 10



Means ± SEM (n=8) denoted with different letters on each day are significantly different ($p < 0.01$)

Figure 11



No significant difference between means ± SEM (n=8) within each dietary group

4.2.2 UDP-Glucuronyltransferase Activity

Following 7 days of dietary treatment, intestinal GT_1 and GT_2 activity were the greatest on the low protein diet, with significant differences observed between the low and the high treatment groups (111-89 and 116-87 nmol/min/mg protein, respectively, $p < 0.01$) as well as between the low and standard treatment groups (111-93 and 116-90 nmol/min/mg protein, respectively, $p < 0.01$) (Tables 6 and 7; Figures 12 and 13). Following 14 days of dietary treatment, GT_1 and GT_2 activity were the greatest on the low protein diet with significant differences observed between the low and high treatment groups (132-94 and 130-96 nmol/min/mg protein, respectively, $p < 0.01$) as well as between the low and standard treatment groups (132-101 and 130-95 nmol/min/mg protein, respectively, $p < 0.01$) (Tables 6 and 7; Figures 12 and 13). On both treatment days no significant differences between the standard and high protein diets were observed.

GT_1 activities in the intestine were significantly different between day 7 and 14 only within the low protein diets (111-132 nmol/min/mg protein, respectively, $p < 0.01$) (Table 6 ; Figure 14). GT_2 activities were significantly different between day 7 and 14 within the low (116-130 nmol/min/mg protein, respectively, $p < 0.01$) and high (87-96 nmol/min/mg protein, respectively, $p < 0.05$) treatment groups (Tables 7; Figure 15).

Table 6: Effect of Dietary Protein on GT_1 Activity: Diet and Day Effect

Tissue	Day	n	Low	Standard	High
Intestine	7	8	$111 \pm 15^{a}_{I}$	$93 \pm 3^{b}_{I}$	$89 \pm 13^{b}_{I}$
	14	8	$132 \pm 13^{a}_{II}$	$101 \pm 8^{b}_{I}$	$94 \pm 13^{b}_{I}$
Kidney	7	8	$27 \pm 2^{a}_{I}$	$25 \pm 6^{a}_{I}$	$36 \pm 9^{b}_{I}$
	14	8	$26 \pm 3^{a}_{I}$	$25 \pm 6^{a}_{I}$	$44 \pm 10^{b}_{I}$
Liver	7	8	$30 \pm 7^{a}_{I}$	$18 \pm 3^{b}_{I}$	$21 \pm 3^{b}_{I}$
	14	8	$32 \pm 7^{a}_{I}$	$19 \pm 2^{b}_{I}$	$19 \pm 4^{b}_{I}$

Diet effect and Day effect: Each value is expressed in nmol/min/mg protein and represents means $\pm$ SD.

Diet effect: Different letter superscripts for each tissue, on each, day 7 and day 14 significantly different ($p < 0.05$).

Day effect: II (subscripts) significantly different from day 7 ($p < 0.01$).

Table 7: Effect of Dietary Protein on GT_2 Activity: Diet and Day Effect

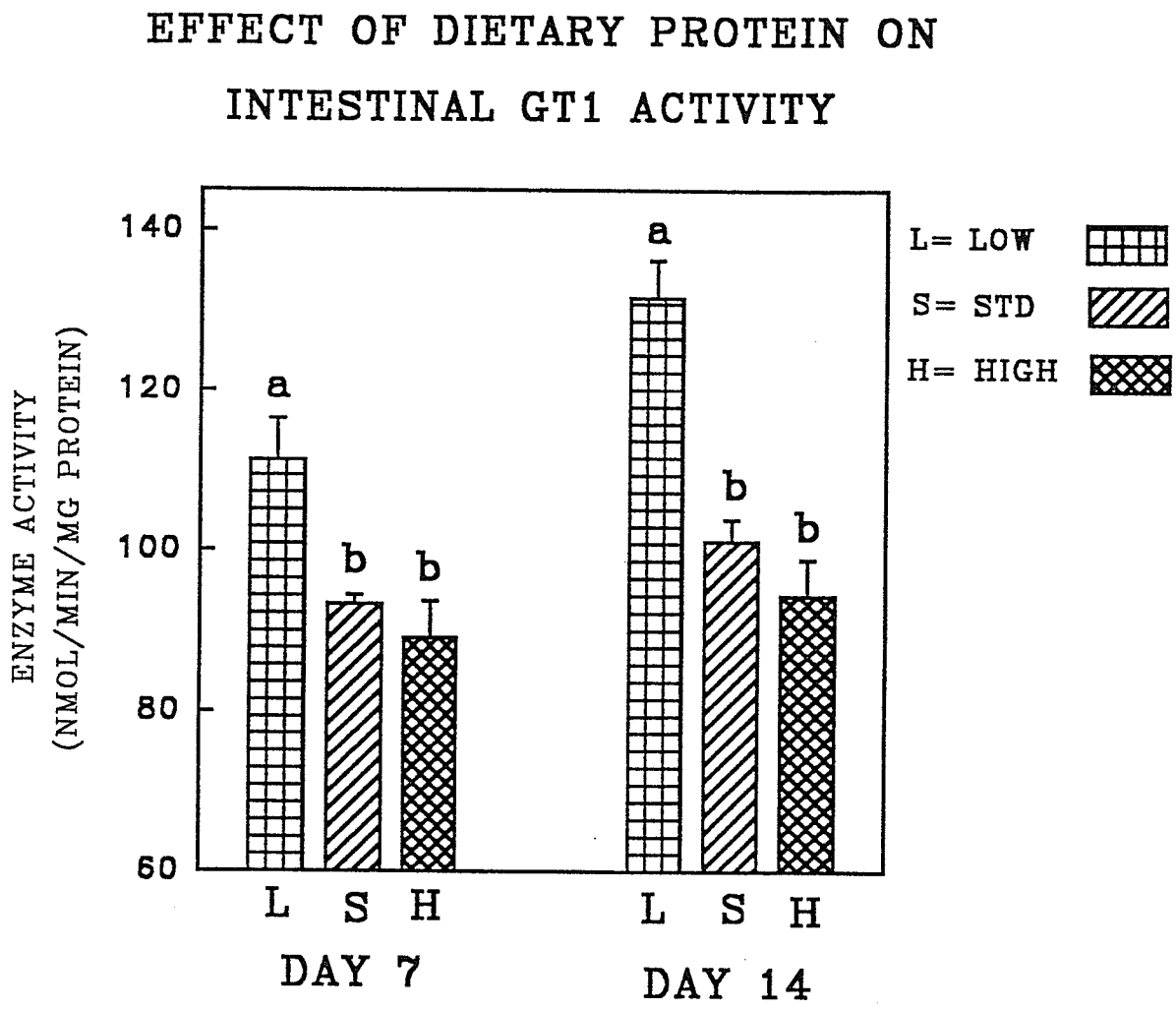
Tissue	Day	n	Low	Standard	High
Intestine	7	8	$116 \pm 12^{a}_{I}$	$90 \pm 7^{b}_{I}$	$87 \pm 11^{b}_{I}$
	14	8	$130 \pm 5^{a}_{II}$	$95 \pm 5^{b}_{I}$	$96 \pm 15^{b}_{II}$
Kidney	7	8	$16 \pm 4^{a}_{I}$	$12 \pm 7^{a}_{I}$	$15 \pm 3^{a}_{I}$
	14	8	$12 \pm 4^{a}_{I}$	$13 \pm 3^{a}_{I}$	$16 \pm 40^{a}_{I}$
Liver	7	8	$18 \pm 4^{a}_{I}$	$12 \pm 2^{a}_{I}$	$12 \pm 2^{a}_{I}$
	14	8	$15 \pm 2^{a}_{I}$	$13 \pm 1^{a}_{I}$	$12 \pm 2^{a}_{I}$

Diet effect and Day effect: Each value is expressed in nmol/min/mg protein and represents means $\pm$ SD.

Diet effect: Different letter superscripts for each tissue on each, day 7 and day 14 significantly different ($p < 0.01$).

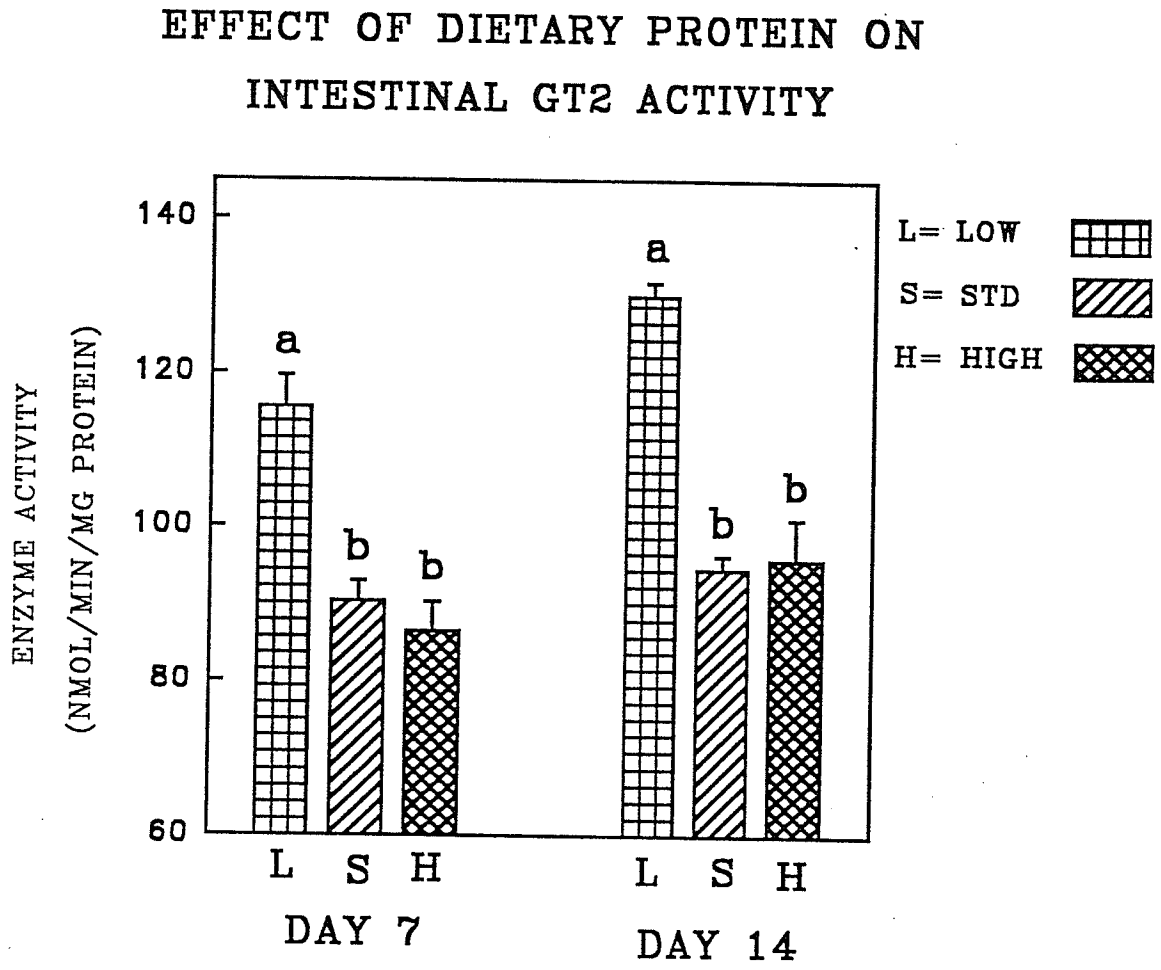
Day effect: II (subscripts) significantly different from day 7 ($p < 0.05$).

Figure 12



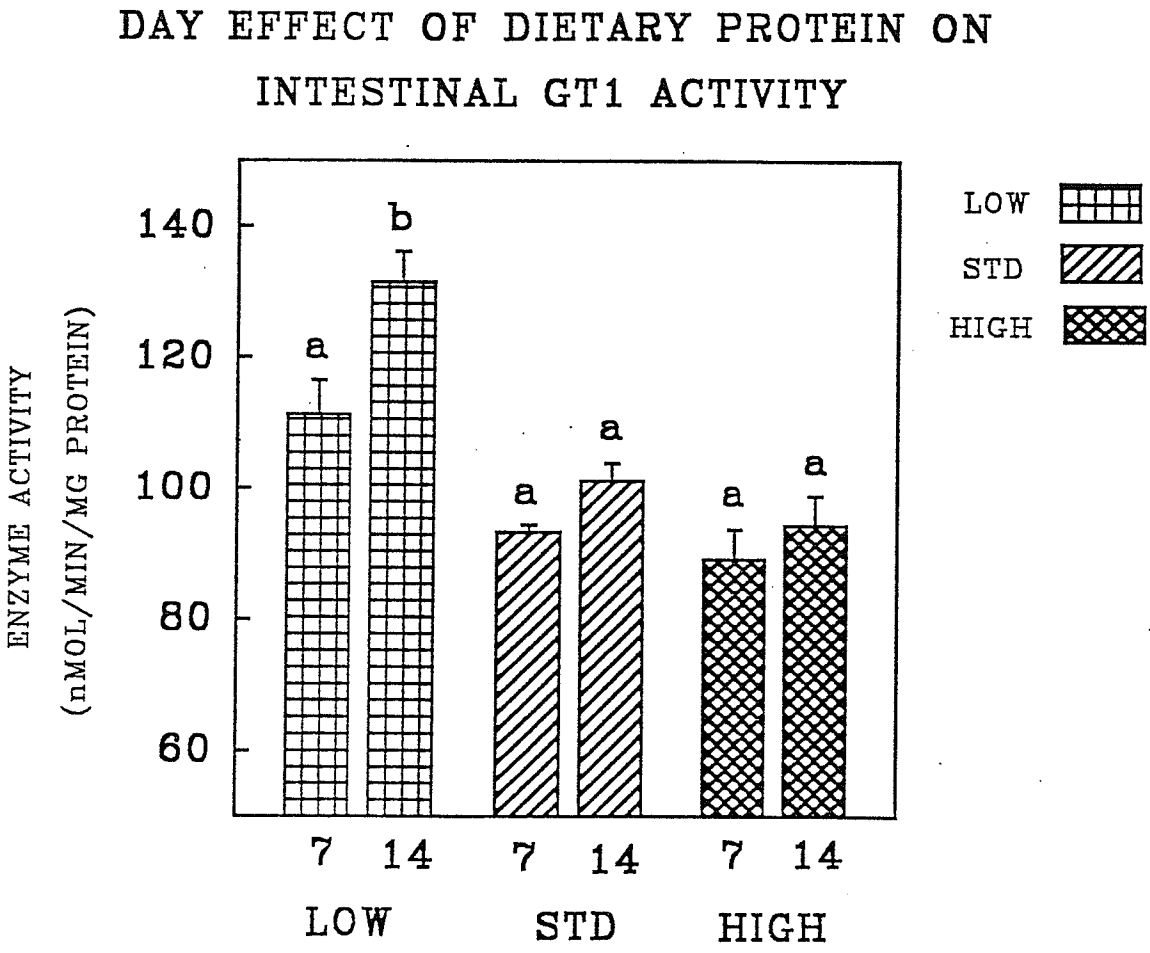
Means ± SEM (n=8) denoted with different letters on each day are ssignificantly different ($p < 0.01$)

Figure 13



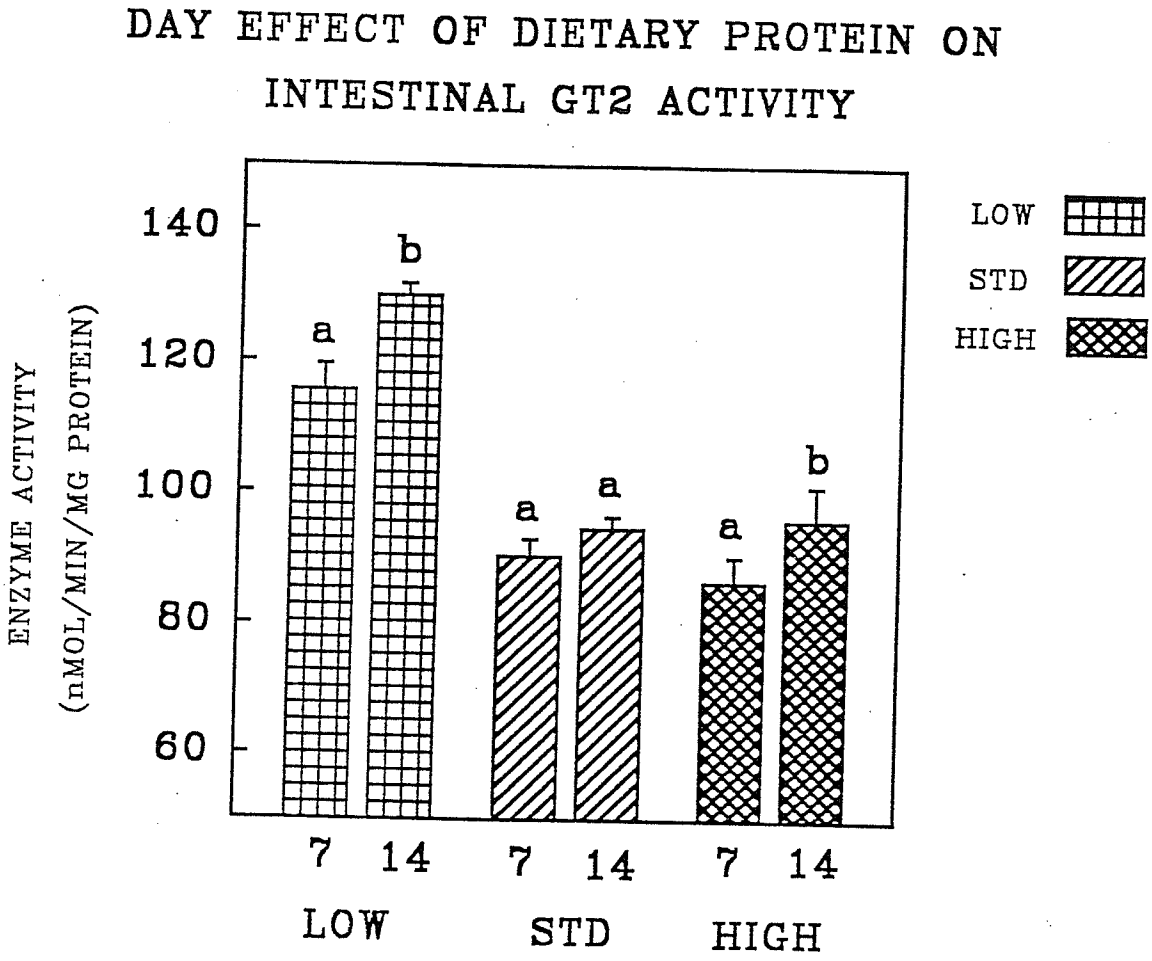
Means ± SEM (n=8) denoted with different letters on each day are significantly different ($p < 0.01$)

Figure 14



Means ± SEM (n=8) denoted with different letters within each dietary group are significantly different ($p < 0.01$)

Figure 15



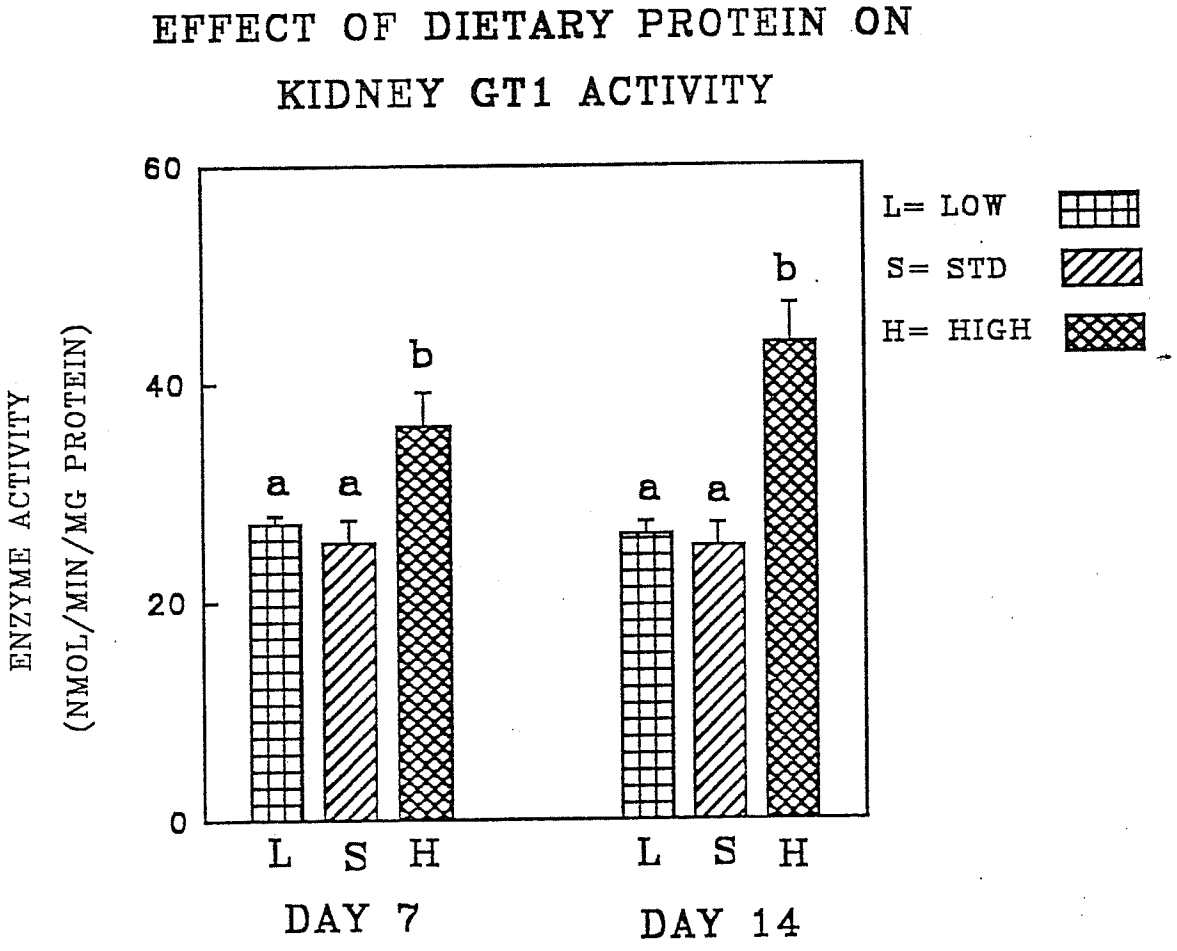
Means ± SEM (n=8) denoted with different letters within each dietary group are significantly different ($p < 0.05$)

Following 7 days of dietary treatment, kidney GT_1 activity was the greatest on the high protein diet, with significant differences observed between the low and the high treatment groups (27-36 nmol/min/mg protein, respectively, $p < 0.05$) as well as between the standard and the high treatment groups (25-36 nmol/min/mg protein, respectively, $p < 0.05$) (Table 6; Figure 16). Following 14 days of dietary treatment, GT_1 activity was the greatest on the high protein diet with significant differences observed between the low and high treatment groups (26-44 nmol/min/mg protein, respectively, $p < 0.05$) as well as between the standard and high treatment groups (25-44 nmol/min/mg protein, respectively, $p < 0.05$) (Table 6; Figure 16). On both treatment days no significant differences were observed between the low and standard protein groups.

In contrast, for kidney GT_2 there were no significant differences observed between the low, standard and high protein diets following 7 or 14 days of dietary treatment (16-12-15 and 12-13-16 nmol/min/mg protein, respectively) (Table 7; Figure 17).

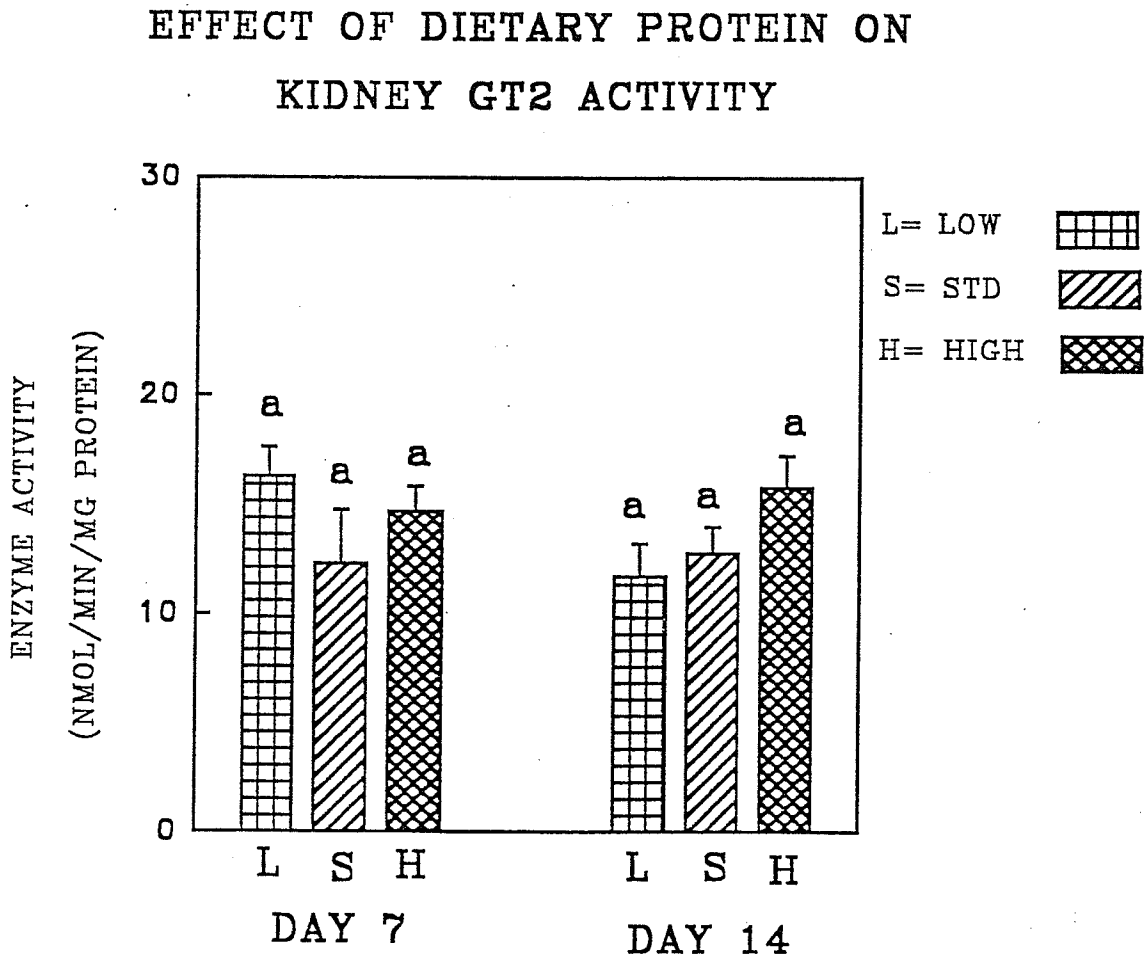
For kidney GT_1 no significant differences between day 7 and day 14 were observed within the low and standard protein groups (Table 6; Figure 18). However, for high protein GT_1 activity was significantly higher after 14 days of treatment compared to 7 days (44/36 nmol/min/mg protein respectively, $p < 0.01$) (Table 6; Figure 18). For GT_2, no significant differences between day 7 and 14 were observed within any dietary treatment (Table 7; Figure 19).

Figure 16



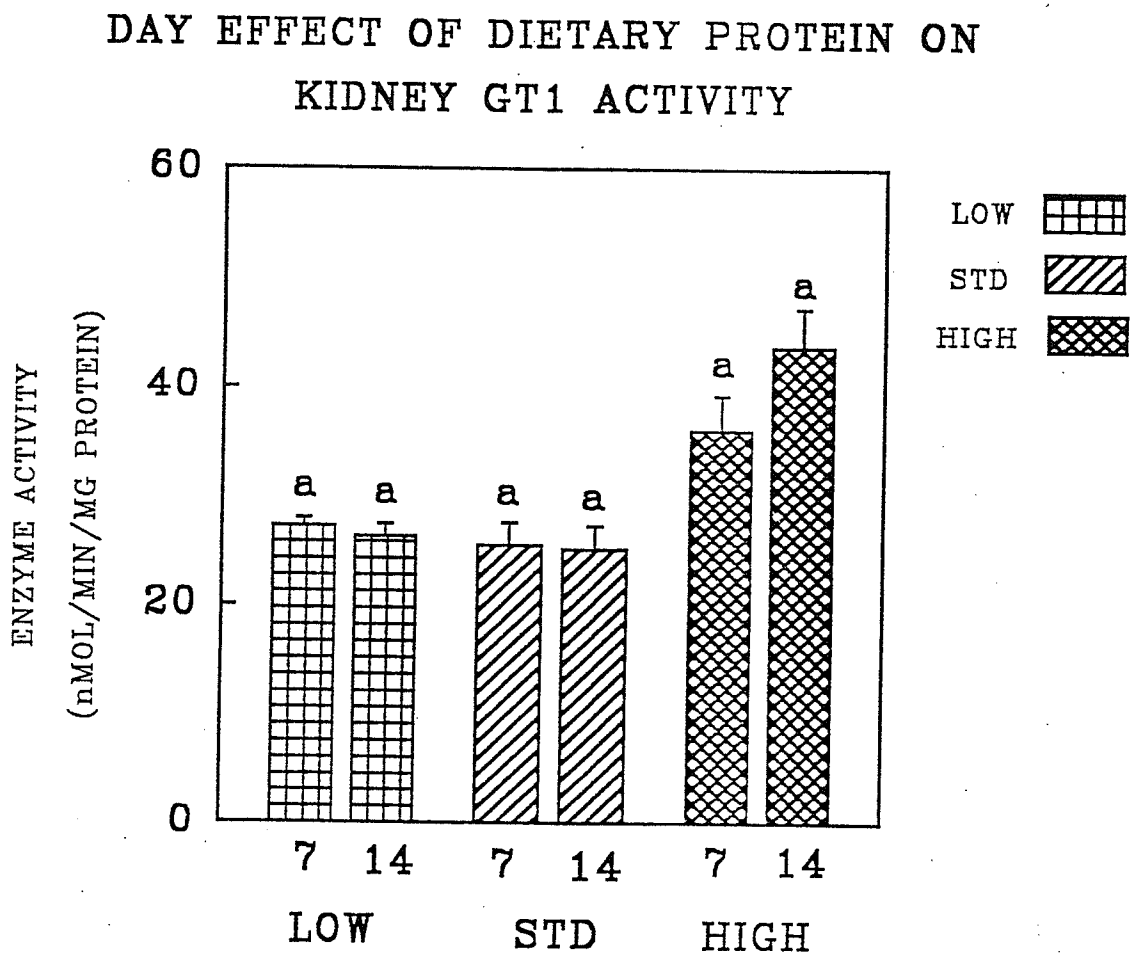
Means ± SEM (n=8) denoted with different letters on each day are significantly different ($p < 0.01$)

Figure 17



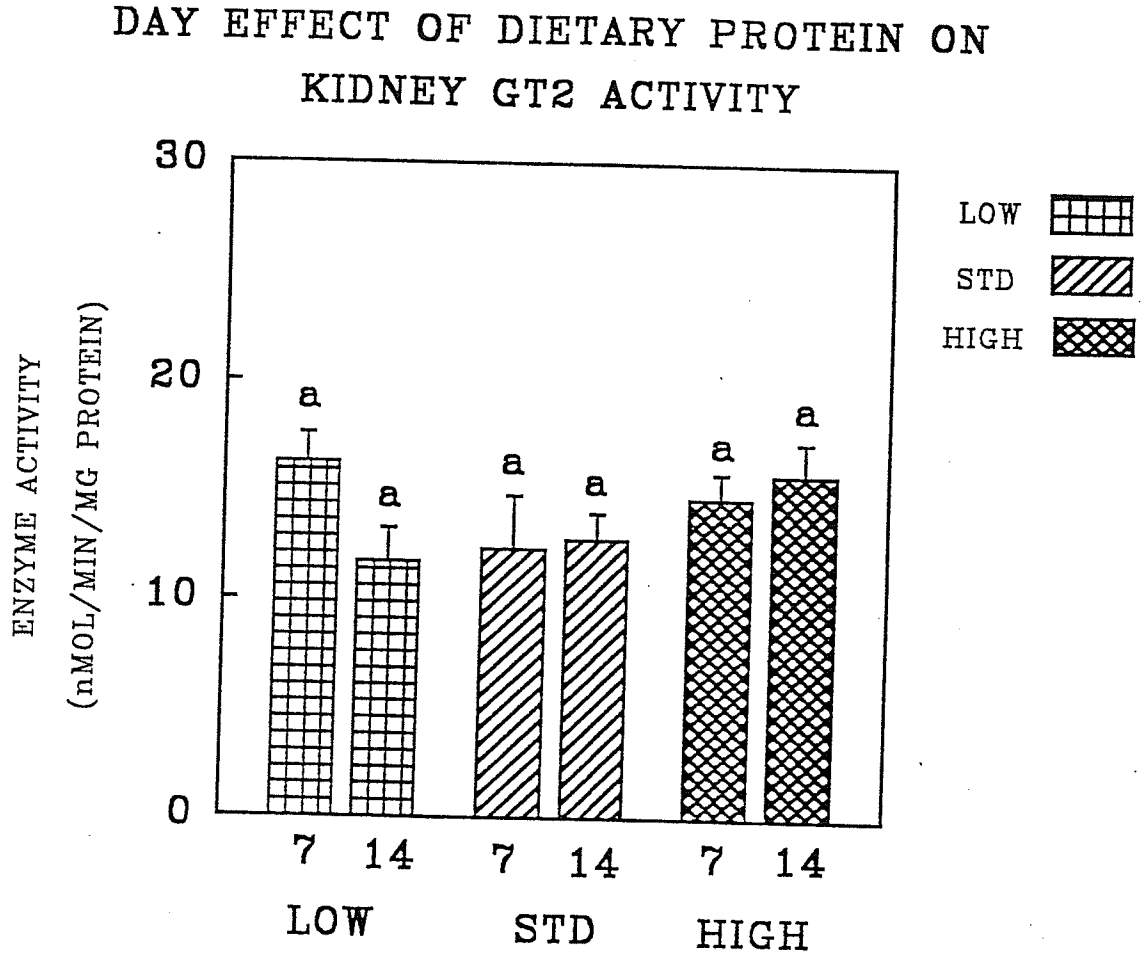
No significant difference between means ± SEM (n=8) on each day

Figure 18



No significant difference between means ± SEM (n=8) within each dietary group

Figure 19



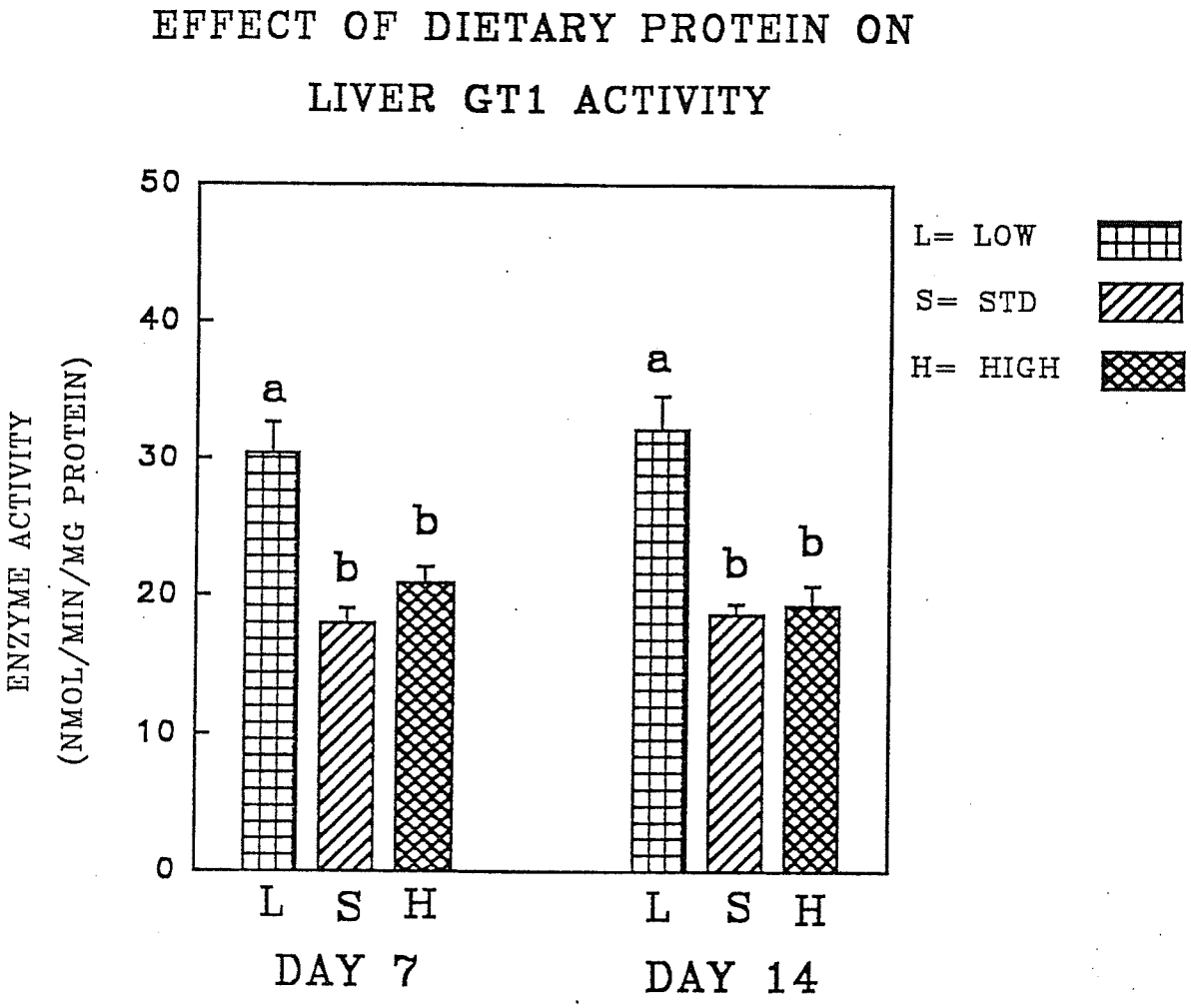
No significant difference between means ± SEM (n=8) within each dietary group

Following 7 days of dietary treatment, hepatic GT_1 activity was the greatest on the low protein diet with significant differences observed between the low and the high treatment groups (30-21 nmol/min/mg protein, respectively, $p < 0.01$) as well as between the low and the standard (30-18 nmol/min/mg protein, respectively, $p < 0.01$) treatment groups (Table 6; Figure 20). Following 14 days of dietary treatment, GT_1 activity was the greatest on the low protein diet with significant differences observed between the low and the high (32-19 nmol/min/mg protein, respectively, $p < 0.01$) treatment groups as well as the low and the standard (32-19 nmol/min/mg protein, respectively, $p < 0.01$) treatment groups (Table 6; Figure 20). On both treatment days, no significant differences between the standard and high treatment groups were observed.

In contrast, for GT_2, no significant differences between the low, standard or high protein diets following 7 or 14 days of dietary treatment were observed (18-12-12 and 15-13-12 nmol/min/mg protein, respectively) (Table 7; Figure 21).

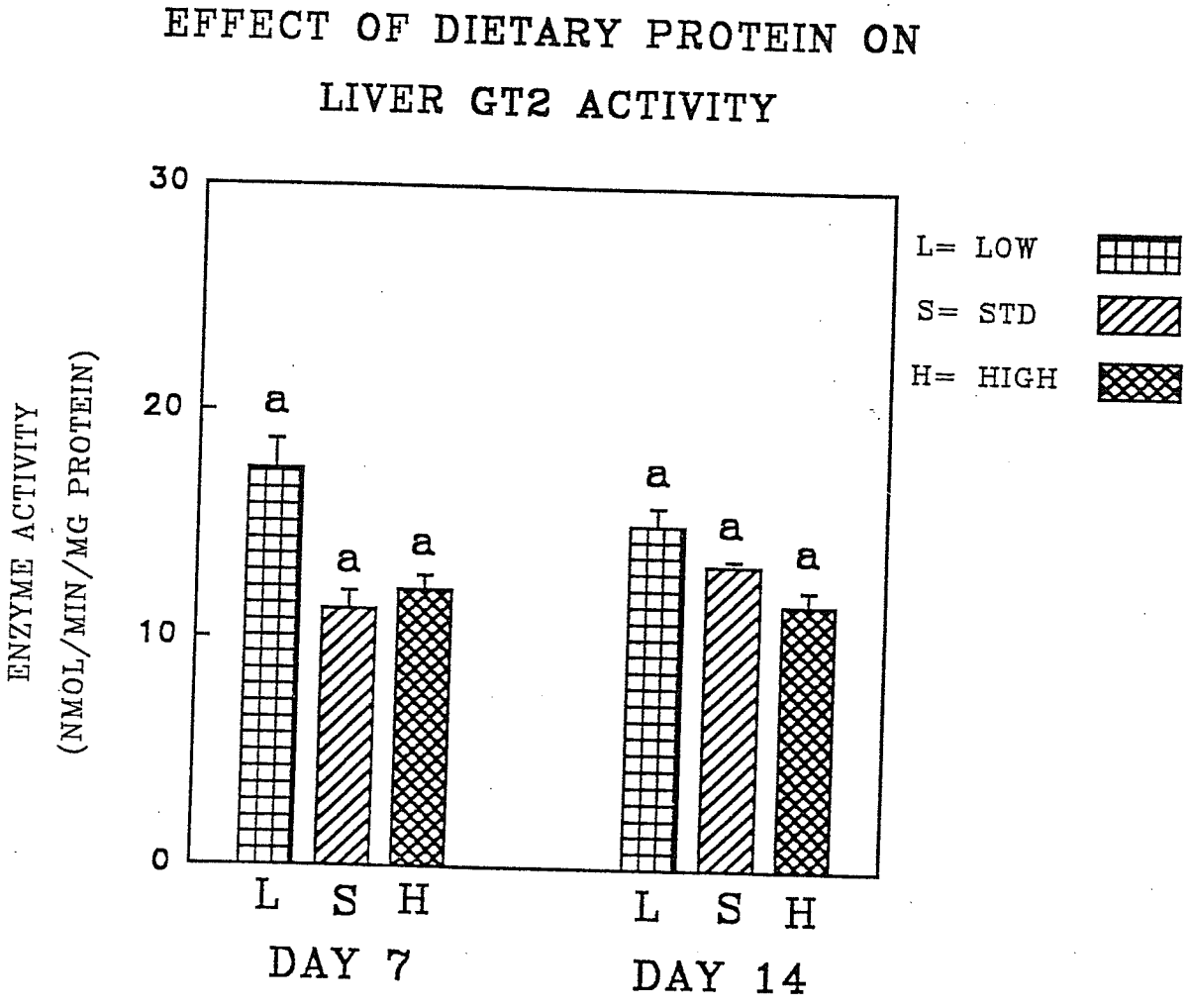
In the liver, no significant differences between day 7 and 14 were observed for GT_1 and GT_2 activities within any dietary treatment (Tables 6 and 7; Figures 22 and 23).

Figure 20



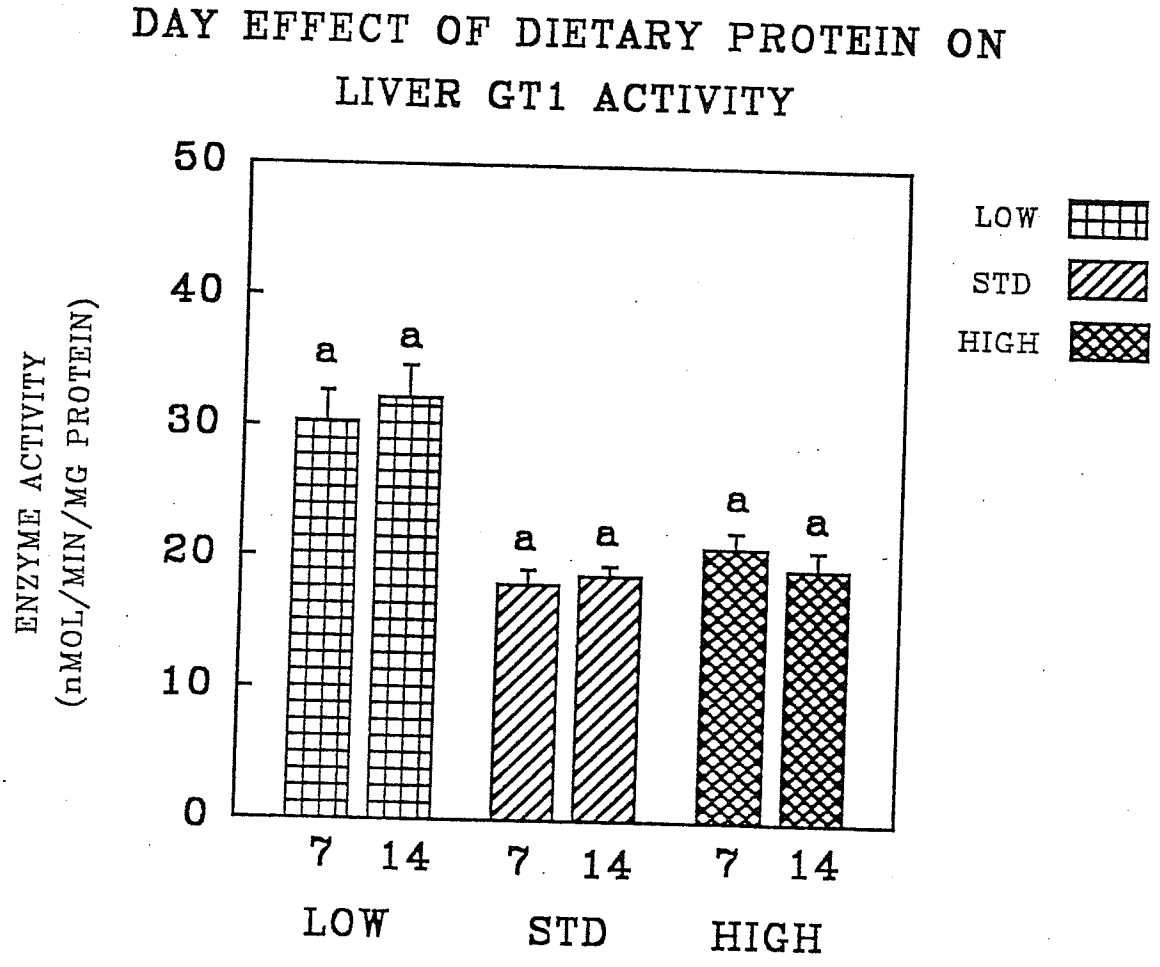
Means ± SEM (n=8) denoted with different letters on each day are significantly different ($p < 0.01$)

Figure 21



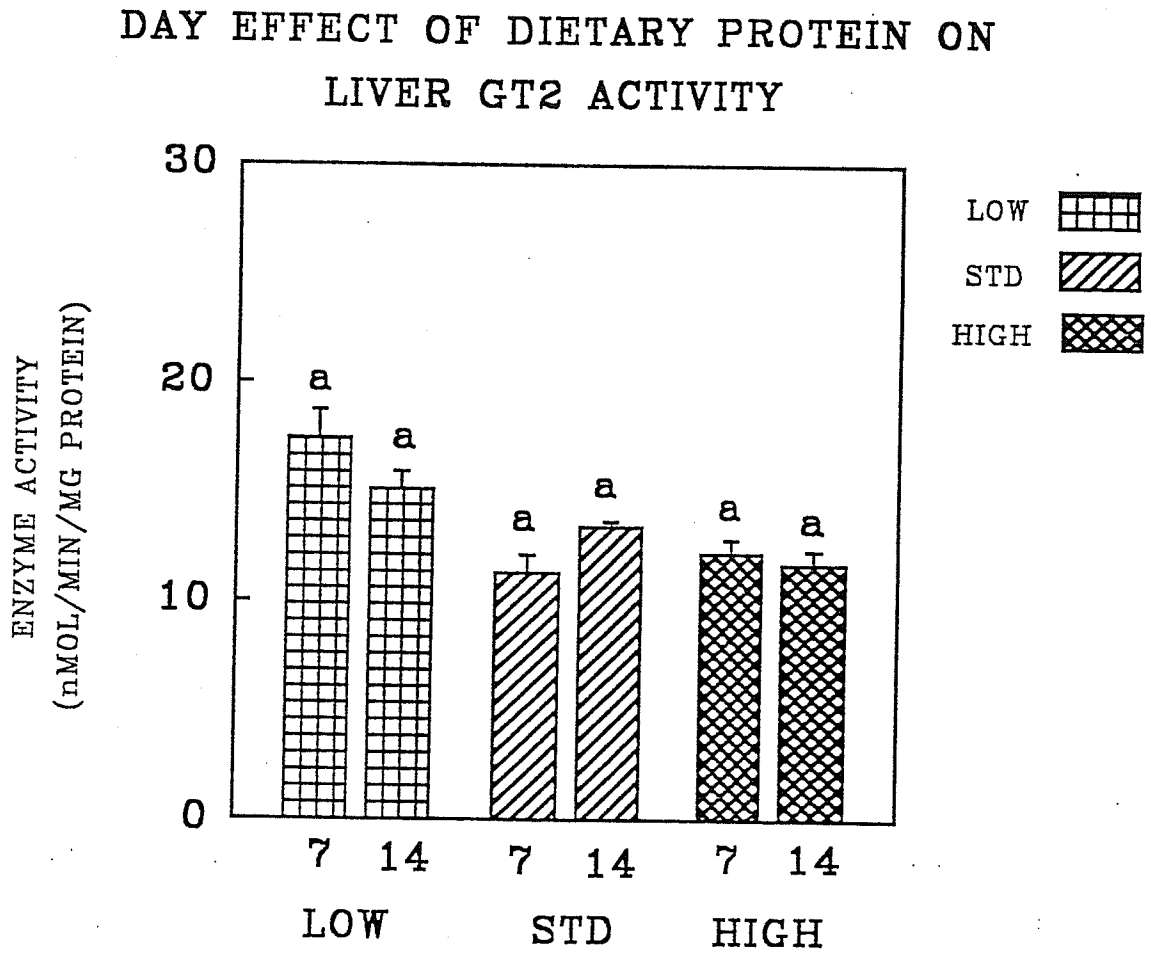
No significant difference between means ± SEM (n=8) on each day

Figure 22



No significant difference between means ± SEM (n=8) within each dietary group

Figure 23



No significant difference between means ± SEM (n=8) within each dietary group

4.2.3 GT_1 Versus GT_2 Activity

Protein diets affected GT isoenzymes differently depending upon the tissue. Following 7 or 14 days of dietary treatment, intestinal GT_1 and GT_2 activities were not significantly different within the low, standard or high dietary protein groups (Table 8; Figures 24 and 25).

Following 7 days of dietary treatment, kidney GT_1 and GT_2 activities were significantly different within the low (27-16 nmol/min/mg protein, respectively, $p < 0.01$), standard (25-12 nmol/min/mg protein, respectively, $p < 0.01$) and high (36-15 nmol/min/mg protein respectively, $p < 0.01$) dietary protein groups (Table 8; Figure 26). Following 14 days of dietary treatment GT_1 and GT_2 activities were significantly different within the low (26-12 nmol/min/mg protein, respectively, $p < 0.01$), standard (25-13 nmol/min/mg protein respectively, $p < 0.01$) and high (44-16 nmol/min/mg protein, respectively, $p < 0.01$) dietary protein groups (Table 8; Figure 27).

Following 7 days of dietary treatment, hepatic GT_1 and GT_2 activities were significantly different within the low (30-18 nmol/min/mg protein respectively, $p < 0.01$) and high (21-12 nmol/min/mg protein, respectively, $p < 0.05$) dietary protein groups (Table 8; Figure 28). Following 14 days, hepatic GT_1 and GT_2 activities were significantly different within the low (32-15 nmol/min/mg protein, $p < 0.01$) and high (19-12 nmol/min/mg protein, $p < 0.1$) dietary protein groups (Table 8; Figure 29). On both days GT_1 and GT_2 activities were not significantly different on standard protein diets.

Table 8: Comparison of GT_1 versus GT_2 Activity in the intestine, kidney and liver.

Tissue	Day	n	GT	Low	Standard	High
Intestine	7	8	GT_1	111 ± 15^a	93 ± 3^a	89 ± 13^a
			GT_2	116 ± 12^a	90 ± 7^a	87 ± 11^a
Intestine	14	8	GT_1	132 ± 13^a	101 ± 8^a	94 ± 13^a
			GT_2	130 ± 5^a	95 ± 5^a	96 ± 15^a
Kidney	7	8	GT_1	27 ± 2^a	25 ± 6^a	36 ± 9^a
			GT_2	16 ± 4^b	12 ± 7^b	15 ± 3^b
Kidney	14	8	GT_1	26 ± 3^a	25 ± 6^a	44 ± 10^a
			GT_2	12 ± 4^b	13 ± 3^b	16 ± 4^b
Liver	7	8	GT_1	30 ± 7^a	18 ± 3^a	21 ± 3^a
			GT_2	18 ± 4^b	12 ± 2^a	12 ± 2^b
Liver	14	8	GT_1	32 ± 7^a	19 ± 2^a	19 ± 4^a
			GT_2	15 ± 2^b	13 ± 1^a	12 ± 2^a

Each value is expressed in nmol/min/mg protein and represents means $\pm$ SD.

For each day and tissue, b suggests GT_2 significantly different from GT_1 ($p < 0.05$).

Figure 24

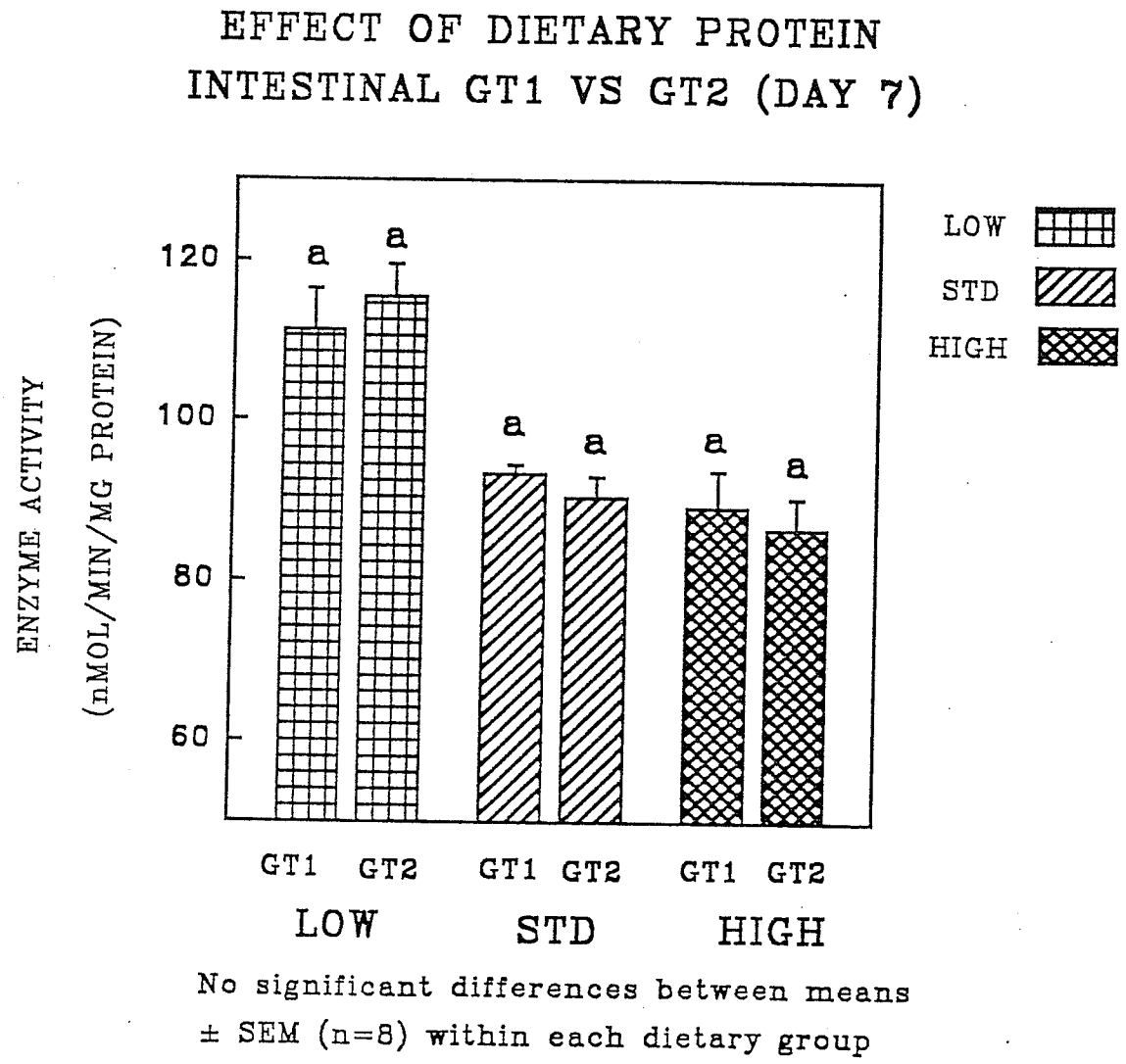
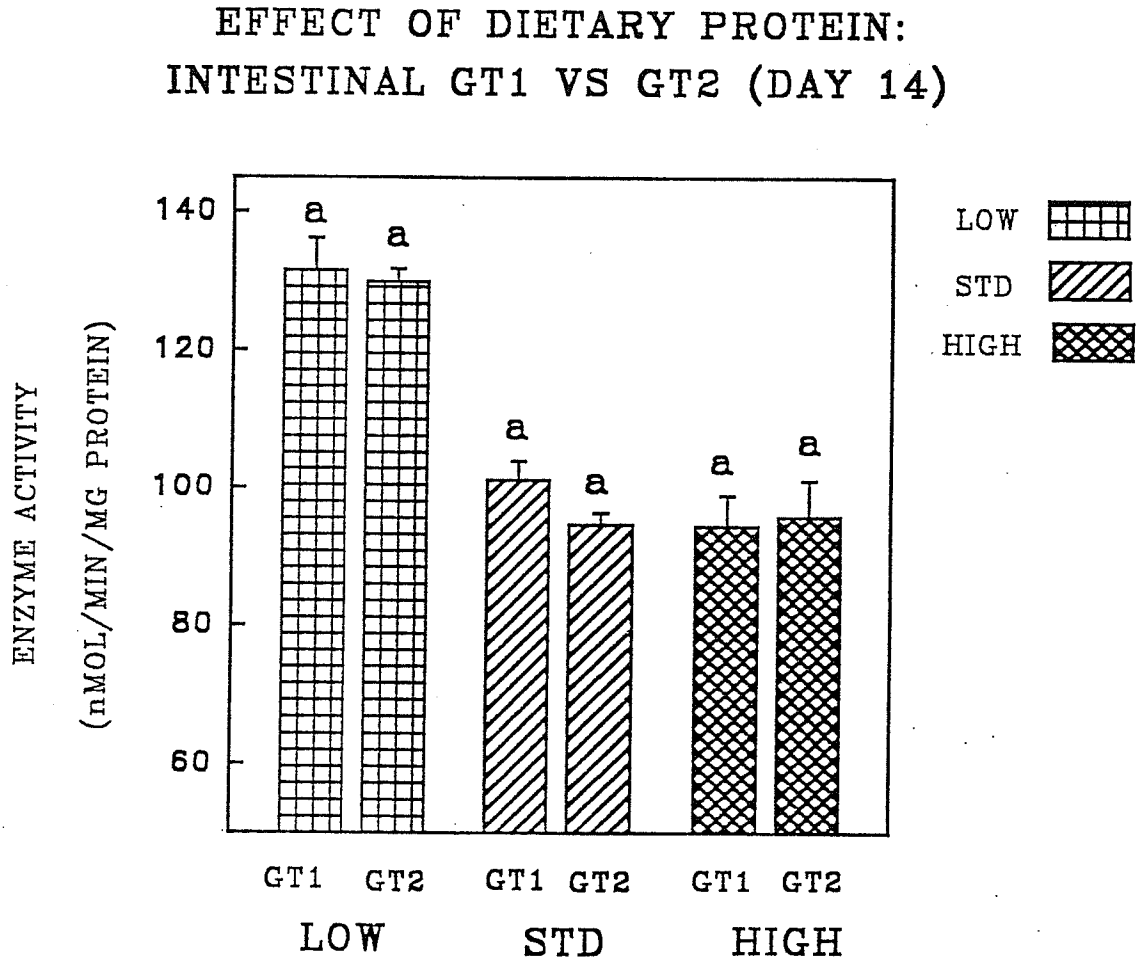
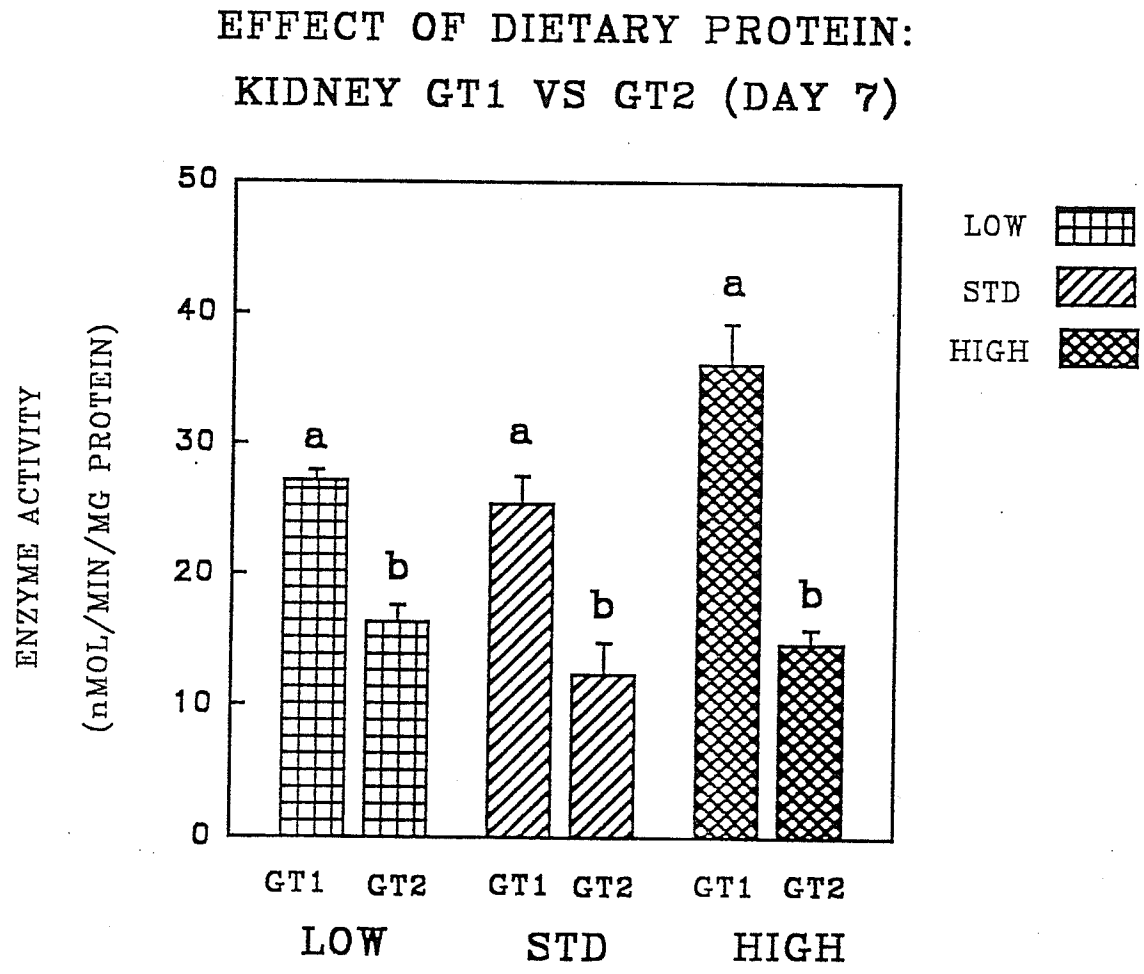

EFFECT OF DIETARY PROTEIN
INTESTINAL GT1 VS GT2 (DAY 7)
ENZYME ACTIVITY
(nMOL/MIN/MG PROTEIN)
120
100
80
60
a
a
a
a
a
a
LOW
STD
HIGH
GT1 GT2 GT1 GT2 GT1 GT2
LOW STD HIGH
No significant differences between means
± SEM (n=8) within each dietary group

Figure 25



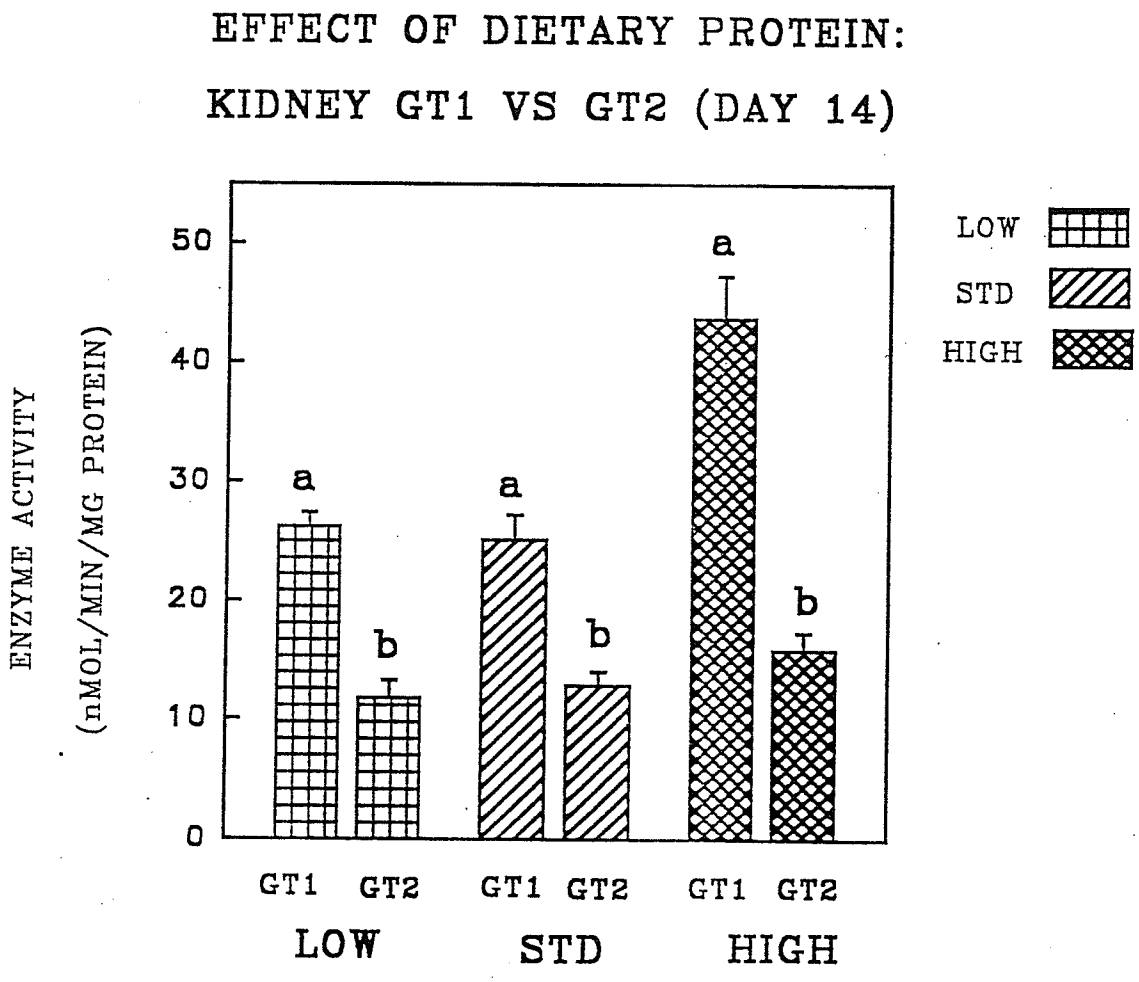
No significant differences between means
± SEM (n=8) within each dietary group

Figure 26



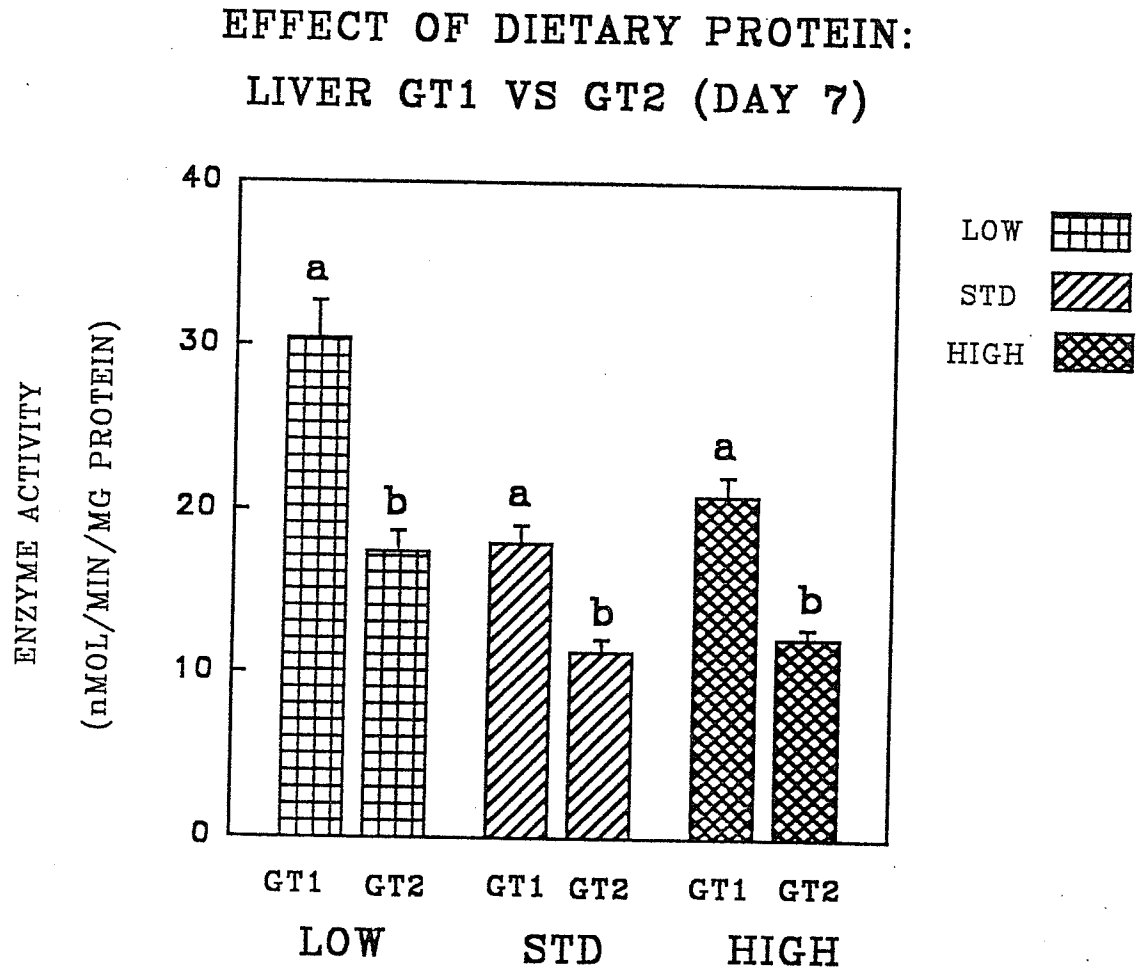
Means ± SEM (n=8) denoted with different letters within each dietary group are significantly different ($p < 0.01$)

Figure 27



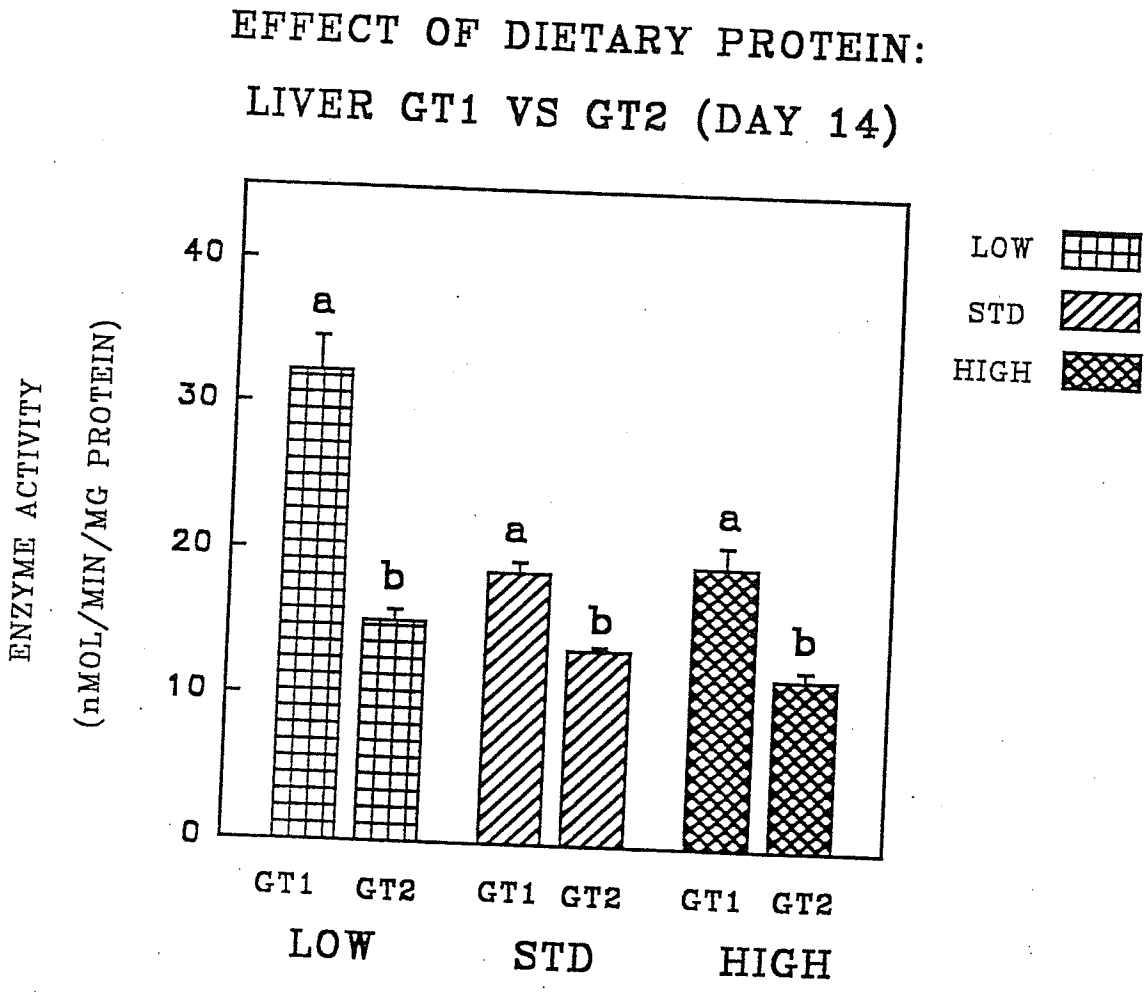
Means ± SEM (n=8) denoted with different letters within each dietary group are significantly different ($p < 0.01$)

Figure 28



Means ± SEM (n=8) denoted with different letters within each dietary group are significantly different ($p < 0.05$)

Figure 29



Means ± SEM (n=8) denoted with different letters within each dietary group are significantly different ($p < 0.01$)

4.2.4 Phase I Versus Phase II Enzymes

In the intestine results for GT_1 and GT_2 (Phase II enzymes) contrast with results for reductase (Phase I enzyme) activity. While reductase, a phase I enzyme, was significantly the greatest on the high protein diet, GT_1 and GT_2 were significantly the greatest on the low protein diet. Significant differences in reductase activity were observed between standard and the high protein diets in contrast to the results for GT_1 and GT_2. Again, in contrast to GT_1 and GT_2 there were no significant differences in reductase activity between low and standard protein diets.

Intertissue Comparison:

While reductase activity was consistently the highest in the intestine, kidney and liver on the high protein diet, GT enzymes responded varyingly to the protein diets in the kidney, liver and intestine.

Chapter V

DISCUSSION

5.1 Animal Weight

The weight gains observed are consistent with our previous work and much of the literature (Merrill and Bray, 1982; Swick and Gribskov, 1983; Semon et al, 1987). In contrast, Hietanen (1980) and Tutelyan et al (1990) report that animals on low protein diets show the least weight gain when compared to the high and standard protein diets. Discrepancies among the various studies could be due to differences in initial weights, duration of the study and the definition of a low protein diet.

Hietanen (1980) and Tutelyan et al (1990) used 80-90g animals, approximately 28 days old (NRC, 1978), while Merrill and Bray (1982) and Fitzpatrick et al (1988) used weanling rats (50-60g), approximately 20 days old (NRC, 1978). Age differences affect the susceptibility to protein deficiency and may explain conflicts in the literature (Merrill and Bray, 1982).

Hietanen et al (1980) and Tutelyan et al (1990) fed protein diets for 4-8 weeks while the Merrill and Bray (1982) and Fitzpatrick et al (1988) studies were of 2 week duration. Long term feeding may result in adaptive responses which increase food intake in the high dietary protein group. This would lead to a greater weight gain than animals on the low protein diet. These conclusions are supported by Moundras et al (1993) who observed that during the first days of high protein diets amino acid metabolism was not fully adapted and protein served as an appetite depression signal in male rats (150-160g). Following 8 days of metabolic adaptation, food intake in the high protein group was

similar to the control rats fed a 15% casein diet. A similar adaptation by the animals on the high protein diet in the Hietanen (1980) and Tutelyan et al, (1990) studies could explain the inconsistencies in the literature.

5.2 Enzyme Assays

It should be noted that no studies could be identified in the current review of literature that simultaneously examined the relationship between dietary effects and temporality.

5.2.1 Reductase: Diet Effect

Little information is available on the effect of dietary protein on intestinal and kidney C-P450 activity. In our study, high protein diets in both the intestine and kidney induced C-P450 activity, while low protein diets did not affect enzyme activity. These results suggest that reductase enzyme in extrahepatic tissue may be characterised as increasing in response to high dietary protein. This could be toxicologically significant, since, it demonstrates that extrahepatic tissues respond in unison to changes from adequate protein status. For a future study it would be interesting to determine if reductase activity responds in a similar fashion in the lungs.

In the liver, reductase activity decreased significantly with decreasing levels of dietary protein. These results are consistent with some of the literature (Clinton et al, 1977; Merrill and Bray, 1982; and Tutelyan et al, 1990). However, Hietanen (1980) observed that hepatic C-P450 was unaffected among the three dietary treatments.

Differences in the literature can be explained by differences in animal age. Hietanen (1980) used older animals while we used weanling animals. Age differences affect susceptibility to protein deficiency with younger rats being more susceptible to dietary deficiencies (Merrill and Bray, 1982).

Toxicological Significance

The toxicological significance of the lower reductase activity observed on the low as compared to the high protein groups can be described generically in terms of two scenarios:

(1) Parent compound is more toxic than the metabolic products. In such cases, protein deficiency would increase the observed toxicity by reducing metabolism and clearance. Many pesticides, such as chlorinated hydrocarbons (DDT), are examples of compounds showing increased toxicity during protein deficiency (Boyd and Krupa, 1970). A high protein diet which increases enzyme activities, as seen in our study, may exert a protective effect against toxicity of such compounds. This hypothesis requires further investigation.

(2) Parent compound is less toxic than metabolic product, that is, the xenobiotic is metabolized to a more toxic product. In such cases, protein deficiency may decrease the observed toxicity. For example, protein deficiency decreases carbon tetrachloride toxicity (McLean and McLean, 1966). A high protein diet which increases enzyme activities, as seen in our study, may not be beneficial in such cases. This hypothesis also requires further investigation.

Intertissue Comparison:

We examined the effect of protein modulations on hepatic and extrahepatic tissues simultaneously and demonstrated unique tissue responses. Extrahepatic tissues did not respond like the liver, suggesting that generalizations found in the literature regarding metabolic response to diet based solely on hepatic studies may be erroneous. Our results support Philpot's (1991) suggestion that extrahepatic tissues are not simply attenuated versions of hepatic tissues.

In our study, when protein status changed from adequate to inadequate, there was a significant reduction in hepatic reductase activity, an observation not seen in the kidney and intestine. In such conditions of reduced hepatic reductase activity, the importance of extrahepatic detoxification may increase. The significance of this extrahepatic metabolism can be demonstrated in terms of dimethylnitrosamine induced toxicity. Nitrosamines are potent carcinogens that are formed when nitrates are converted to nitrites and onto nitrosamines. Biotransformation of dimethylnitrosamine (found in cured meat products) involves bioactivation to a methylating agent by hepatic and kidney C-P450 enzymes (Dekant and Vamvakas, 1992). Lowered hepatic reductase activity due to low protein diets could lower the extent of metabolic activation in the liver, permitting more carcinogen to reach the kidney. Since kidney reductase activity was not significantly different between the standard and low protein diets, the kidney may be able to handle the increase toxic load. This hypothesis requires further investigation.

5.2.2 Reductase: Day Effect

The relationship between dietary effects and temporality has not been previously examined. In our study, kidney and liver reductase activity did not change between day 7 and 14 within any of the dietary treatments. This suggests that once the level of reductase has been established by dietary protein modulation, the effects of protein diets in the kidney and liver do not compound over time for at least fourteen days. Our results are a snap shot over a 14 day period. In the absence of supporting evidence, these results are too limited to establish long or short term consequences with any certainty. A future study could determine if these enzyme profiles are changed by protein modulation beyond 14 days.

The reduced reductase activity in the intestine of the control animals after 14 days, could be an age related effect, similar to the observations of Chenglis et al (1988) in the liver. We used weanling animals (66 g) corresponding to an approximate age of 25 days (NRC, 1978). By the end of the experimental period the animals were approximately 50 days old; an age at which Chenglis et al (1988) reported declining hepatic reductase activity. The absence of an age related effect in the intestine on the low and high protein diets suggests an adaptive response by the intestine to the dietary protein supply. This adaptive response by the intestine reinforces the suggestions that the intestine functions as the body's first line of defence (Hoensch and Hartman, 1981).

5.2.3 UDPGT: Diet Effect

In our study, GT_1 activity in the kidney on both day 7 and 14 was the greatest on a high protein diet. For GT_2 there were no significant differences between the three treatments on both day 7 and 14. The significance of this is unclear since little information is available in the literature on the effects of protein states on kidney detoxification systems.

The higher hepatic and intestinal UDPGT activities observed on a low protein diet are consistent with the literature (Catania and Carrillo, 1990; Wood and Woodcock 1970; 1971; Graham et al, 1974; Hietanen 1980; and Merrill and Bray, 1982). The higher UDPGT activities on the low protein diets suggest an adaptive response of the detoxification mechanisms that compensate for the unfavourable conditions, in both the intestine and liver. The absence of an adaptive response by UDPGT enzymes on high protein diets in both the liver and intestine, suggests that UDPGT enzymes in these tissues are induced when protein states change from standard to low and remain unchanged when dietary protein increases above the requirement.

Tutelyan et al (1990) study is the only study identified in the current review of literature that examined the effect of protein diets on UDPGT isoenzymes. Our results for intestinal GT_1 and GT_2 and hepatic GT_1 are consistent with the observations of Tutelyan et al (1990). However, they observed a decrease in GT_2 activities on a low protein diet, while we observed no significant differences between the three dietary treatments. The difference in observed results could be due to the varying length of the studies. The extended protein deficient state (60 days) in the Tutelyan et al (1990) study

may have resulted in reduced GT_2 activities on a low protein diet. The fourteen days duration of our study may not be enough time to bring about disparate levels of GT_1 and GT_2 on a particular protein diet. For a future study it would be interesting to determine if extended periods of protein deficiency (> 14 days) could alter the responses of individual GT isozymes on a given protein status. Therefore, it is important to note that while differing protein states alter the individual responses of UDPGT isoenzymes, the duration of the protein deficiency for a given diet may draw out disparate levels of the isoenzymes.

Mechanism For Altered UDPGT Enzyme Activity on Low Protein Diets

Investigators such as Wood and Woodcock (1970; 1971), Graham et al (1974) and Hietanen (1980) have suggested that low protein diets alter hepatic UDPGT activity at the membrane level via alterations of the phospholipid composition of the membrane. Both Catania and Carrillo (1990) and Tutelyan et al (1990) have suggested similar mechanisms for the higher UDPGT activity in the intestine on low protein diets. Whether the higher GT_1 activity observed in the kidney on the high protein diet is also related to alterations in the phospholipid components of the membrane, needs to be investigated.

Intertissue Comparison:

While UDPGT activities in the liver and intestine were the greatest on a low protein diet, in the kidney they were the greatest on a high protein diet. In addition, diet significantly modulated GT_1 and GT_2 activity in the intestine, while diet had little or no

effect on GT_2 activity in both the kidney and liver. From the previous discussion it is clear that each tissue is responding uniquely to dietary modulations by proteins and phase II enzyme responses in the kidney and intestine are completely unlike each other. Given these results, it appears that the extent of detoxification of xenobiotics by phase II isozymes at various sites may vary depending on the dietary protein supply.

5.2.4 UDPGT: Day Effect

The effect of protein diets on UDPGT enzymes at different time periods has not been previously studied. This study demonstrated that duration of protein deficiency affects the intestine more than the liver and kidney. This may be explained by the nature of the tissue. The intestine is a dynamic tissue with a high rate of turnover and any changes in nutritional status should be quickly reflected. The higher GT_1 activity on a low protein diet and higher GT_2 activities on low and high protein diets after 14 days emphasizes the fast and continued adaptive response of the intestinal phase II enzymes to changes in protein supply. The higher enzyme activities suggest that alterations in the phospholipid components of the membrane by low protein diets may be compounding over time, a hypothesis needing investigation. Whether the higher GT activities on a high protein diet are also associated with changes in the phospholipid membrane remains unconfirmed. The results in the kidney and liver suggest that once GT_1 and GT_2 levels are induced by protein modulation, continued low and standard protein supply will not affect enzyme levels for at least fourteen days. Although high protein diets in the kidney had some but not a dramatic effect on GT_1 activity, it would be interesting for a future

study to determine if continued high protein diets (> 14 days) could further induce GT_1 activity in the kidney.

Toxicological Significance

The toxicological significance of our UDPGT results, can be demonstrated in terms of a commonly used analgesic, acetaminophen. Acetaminophen, in therapeutically recommended doses, is an effective and safe analgesic that is metabolized to glucuronyl and sulfate conjugates and subsequently eliminated in the urine. A small portion of acetaminophen is metabolized by C-P450 to a reactive intermediate and is consequently inactivated by glutathione (GSH). However, in overdose conditions, the liver's capacity to metabolize acetaminophen is exceeded, forming a larger fraction of the reactive metabolite, thus, depleting GSH. As a result, a larger quantity of unmetabolized drug is delivered to the kidney, increasing the toxic load on the kidney (Prescot and Wright, 1973; Davis et al, 1976). Jones et al (1979) demonstrate that the kidney can metabolize acetaminophen to glucuronyl and sulfate conjugates but its capacity to do so is substantially less than the liver. We suggest that in conditions of acetaminophen overdose, a higher protein content could exert a protective effect, even though a high protein diet could increase the production of reactive intermediates via C-P450 metabolism. This protective effect could result from a concomitant increase in GT_1 activities in the kidney as seen in our study, thus compensating for the increased production of reactive intermediates and thereby reducing the toxic effects of acetaminophen overdose. This hypothesis requires investigation.

5.2.5 GT_1 vs GT_2

This study demonstrates the unique responses of GT enzymes to dietary modulations in each tissue. In the intestine, on both day 7 and 14, there were no significant differences between GT_1 and GT_2 activities within any dietary treatment. In the kidney, on both days, there were significant differences between GT_1 and GT_2 on each level of dietary treatment. Since the differences in GT_1 and GT_2 in the kidney appear in the control as well as in the low and high protein groups, suggests that the differences may not be related to diet. This hypothesis requires investigation. In the liver, on both day 7 and 14 there were significant differences between GT_1 and GT_2 within the low and high protein diets. The fact that the differences in GT_1 and GT_2 did not appear in the control groups, suggests that differences may be diet related in the liver.

5.2.6 Phase I vs phase II

Protein diets had opposite effects on phase I and phase II enzymes in the intestine, an effect that was not seen in any other tissue. This could be significant. Normally, enhanced metabolism of xenobiotics in the intestine, can modify the relative amounts of parent compounds and metabolites that can be transported to other tissues (Gupta et al, 1989). In this way the small intestine may protect other tissues by metabolizing ingested toxic substances and carcinogens to inactive products. However, Gupta et al (1989) and Sipes and Gandolfi (1991) suggest that these protective effects could be far less effective in conditions of contrary responses of phase I and phase II enzymes. Such an opposite response was seen in our study. In the intestine, GT_1 and GT_2 (phase II enzymes) were

the highest on the low protein diet, while similar effects were not seen for reductase - a phase I enzyme. Decreased intestinal biotransformation capacity (phase I) coupled with increased intestinal conjugating capacity (phase II) could allow active metabolites to be produced that may find enough time to react with cellular molecules like DNA and increase susceptibility of tissues to chemical carcinogenesis (Gupta et al, 1989; Sipes and Gandolfi, 1991). However, Gupta et al (1989) suggest that further studies are required to prove such a hypothesis.

CHAPTER VI

CONCLUSION

The effect of protein diets on extrahepatic enzyme systems has not been extensively studied. The objective of this study was to examine the effect of dietary protein on hepatic and extrahepatic tissue. The study demonstrated that hepatic and extrahepatic tissues responded differently to protein modulations.

Hepatic reductase activity decreased significantly with decreasing levels of dietary protein, while in the kidney and intestine it increased on a high protein diet and remained unchanged on standard and low protein diets. UDPGT isoenzymes in the liver and the intestine were the greatest on a low protein diet, while, in the kidney they were the greatest on a high protein diet. GT_1 activity in the intestine, kidney and liver was modulated by dietary protein, while, GT_2 activities were modulated only in the intestine. These results suggest that generalizations about extrahepatic tissues based solely on hepatic studies are erroneous. Our results support Philpot's (1991) suggestion that extrahepatic tissues are not simply attenuated versions of hepatic tissues.

Our study examined the relationship between dietary effects and temporality which has not previously been studied. Duration of protein diets affected reductase activity in the intestine but did not affect it in the kidney and liver. Reductase activity in the intestine of control animals declined after 14 days which may be an age related effect. The absence of this age related effect on the low and high protein diets suggests an adaptive response by the intestine to dietary protein. Duration of protein diets had a greater effect on intestinal UDPGT isoenzyme activity than in the liver and kidney. This

may be explained by the nature of the tissue. The intestine is a dynamic tissue with a high rate of turnover in which changes in nutritional status are quickly reflected.

Reduced hepatic reductase activity on low protein diets (compared to high protein diets) may be explained by breakdown of intracellular protein to meet protein requirements. This may not hold true for the intestine and kidney as seen in our study, where, reductase activity on low protein diets was not significantly different from standard protein diets. A future study could examine the reasons for these differences among tissues. Higher UDPGT activity on low protein diets in the liver and intestine have been related to alterations in phospholipid composition of membrane. Whether the higher UDPGT activities in the kidney on the high protein diets is also related to similar alterations needs to be investigated. The temporal effects observed in our study are a snap shot over a 14 day period. In the absence of supporting evidence, these results are too limited to establish long or short term consequences with any certainty.

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Appendix A

LIST OF MAJOR EQUIPMENT

1. Beckman L5-50B ultracentrifuge
2. ECP-150 Electronic cold plate
3. Fisher Accumet pH meter, model 810
3. Milton Roy spectronic 3000 spectrophotometer
4. Potter-Elvejhem Homogenizer
5. Polytron homogenizer
6. Sp6-300 Spectrophotometer, Pye Unicam

Appendix B

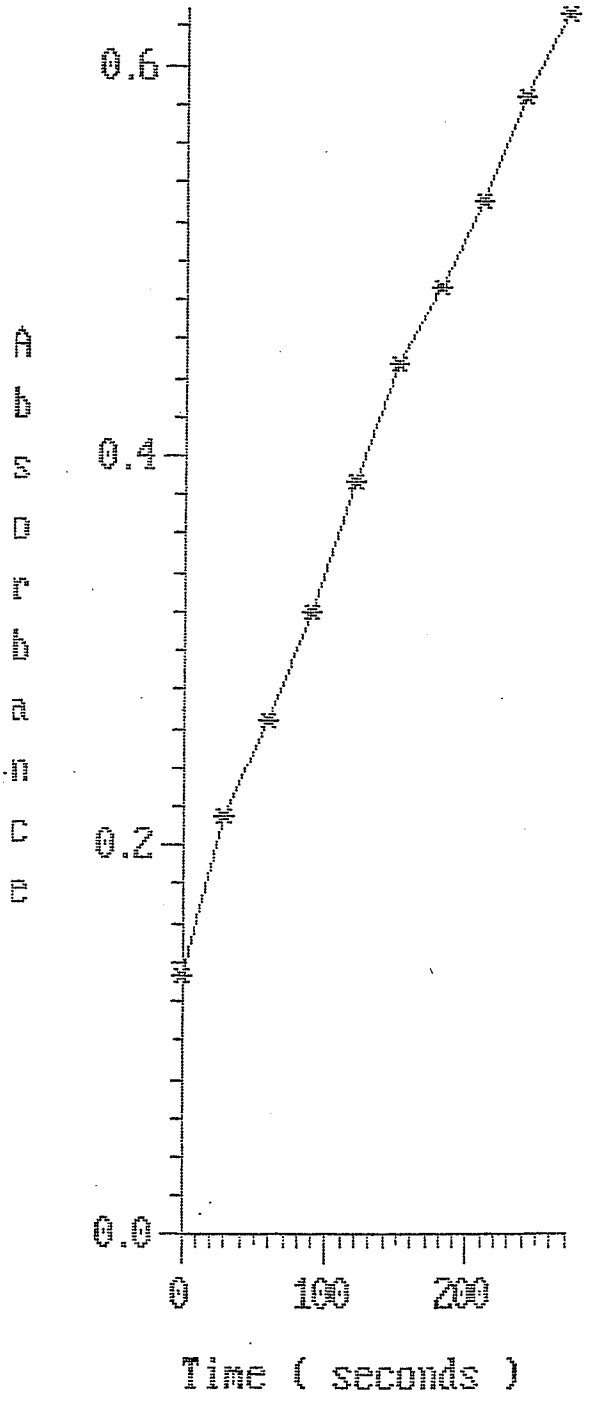
NUTRIENT SUPPLIERS

Table 9

Nutrient	Suppliers
Corn Starch	U of M Food Services
Cellufil (Non Nutritive Bulk)	USB (United States Biochemical) 21000 Miles Parkway, Cleveland OH 44128
Dextrose Sugar	The R. Wine Baril Winnipeg, MB
Vitamin Free Casein	USB
D-L Methionine	USB
Mazola Corn Oil	U of M Food Services
Lard	U of M Food Services
AIN Vitamin Mixture 76	USB
AIN Mineral Mixture 76	USB
Choline Bitarate	USB

Appendix C

OPTIMIZATION OF INTESTINAL REDUCTASE ACTIVITY



Initial Y:	0.136
Final Y:	0.628
Initial Time:	0.000
Final Time:	270.000

Rate:	1.08212e-001	*
Factor:	1106.19	
Calc:	1.19704e+002	**

LINEAR

Y-Intercept: 0.158

Corr. Coef.: 0.997

Std. Dev.: 0.010

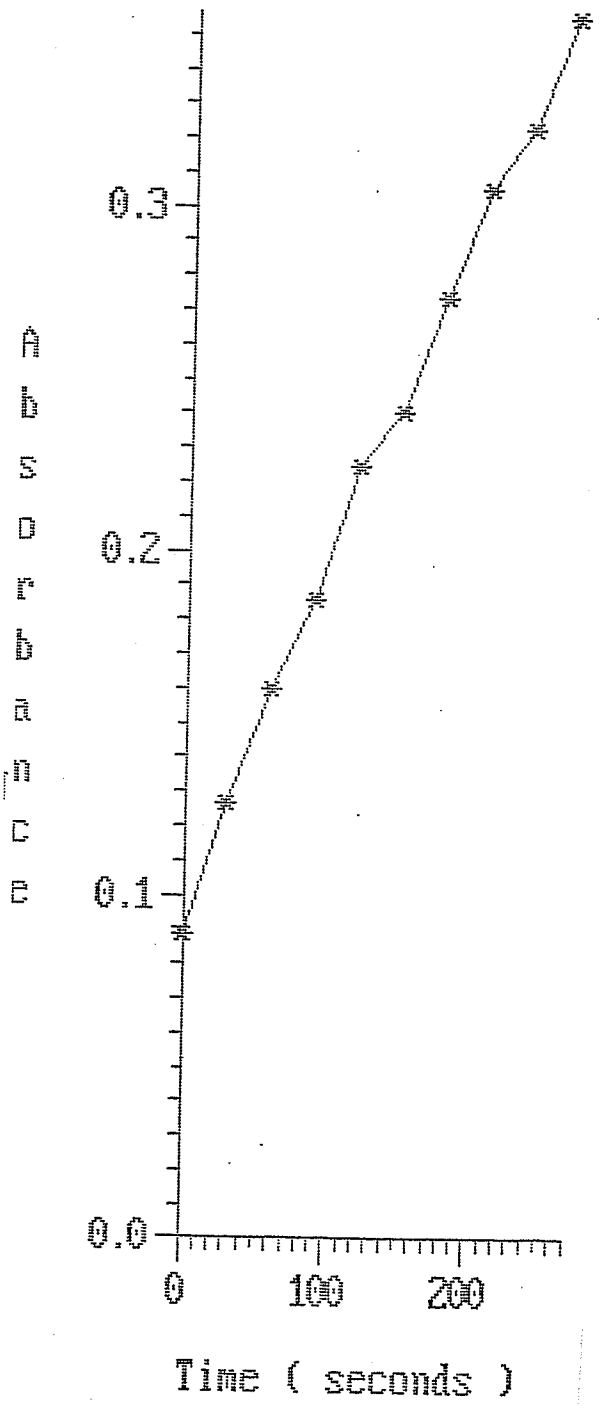
Rate Constant (/min):
2.62875e-001

* Abs/Min

** Abs/min/mg

Appendix D

OPTIMIZATION OF KIDNEY REDUCTASE ACTIVITY



Initial Y: 0.089
Final Y: 0.356
Initial Time: 0.000
Final Time: 270.000

Rate: 5.79360e-002 *
Factor: 1106.19
Calc: 6.40885e+001 **

LINEAR
Y-Intercept: 0.099
Corr. Coef.: 0.998
Std. Dev.: 0.005

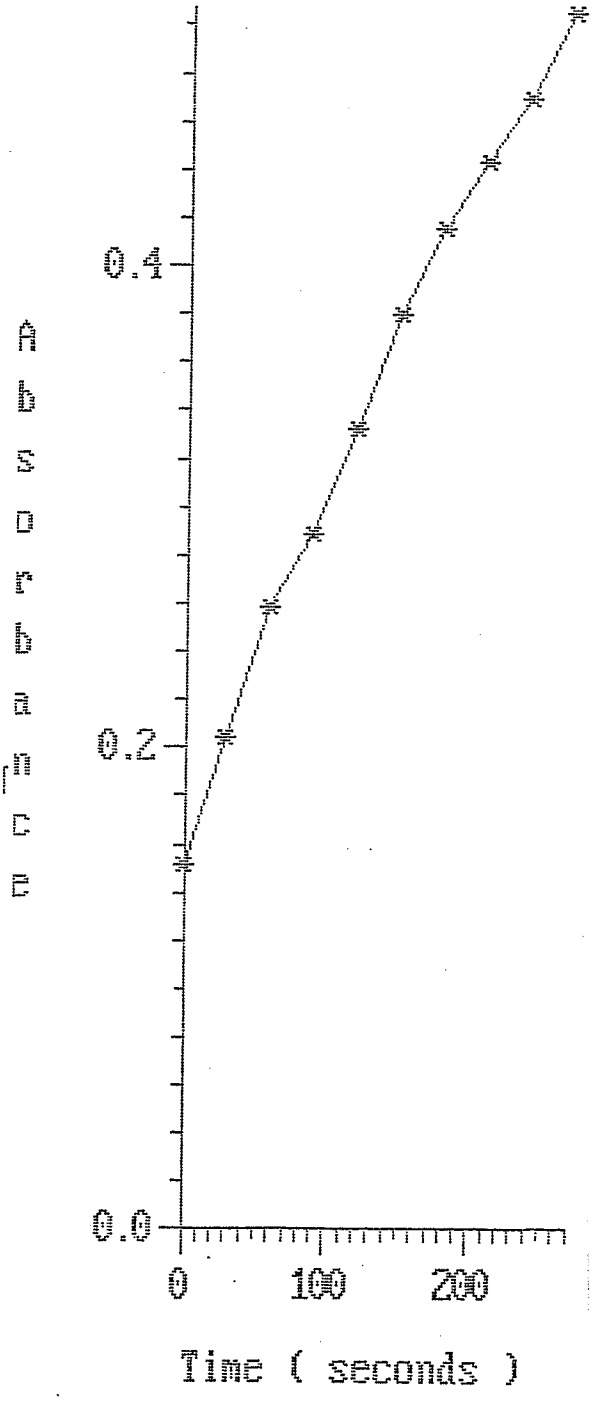
Rate Constant (/min)
2.75135e-001

* Abs/Min

** ABS/MIN/mg

Appendix E

OPTIMIZATION OF LIVER REDUCTASE ACTIVITY



Initial Y: 0.152
Final Y: 0.508
Initial Time: 0.000
Final Time: 270.000

Rate: 7.77725e-002 *
Factor: 884.96
Calc: 6.88252e+001 **

LINEAR

Y-Intercept: 0.172
Corr. Coef.: 0.995
Std. Dev.: 0.010

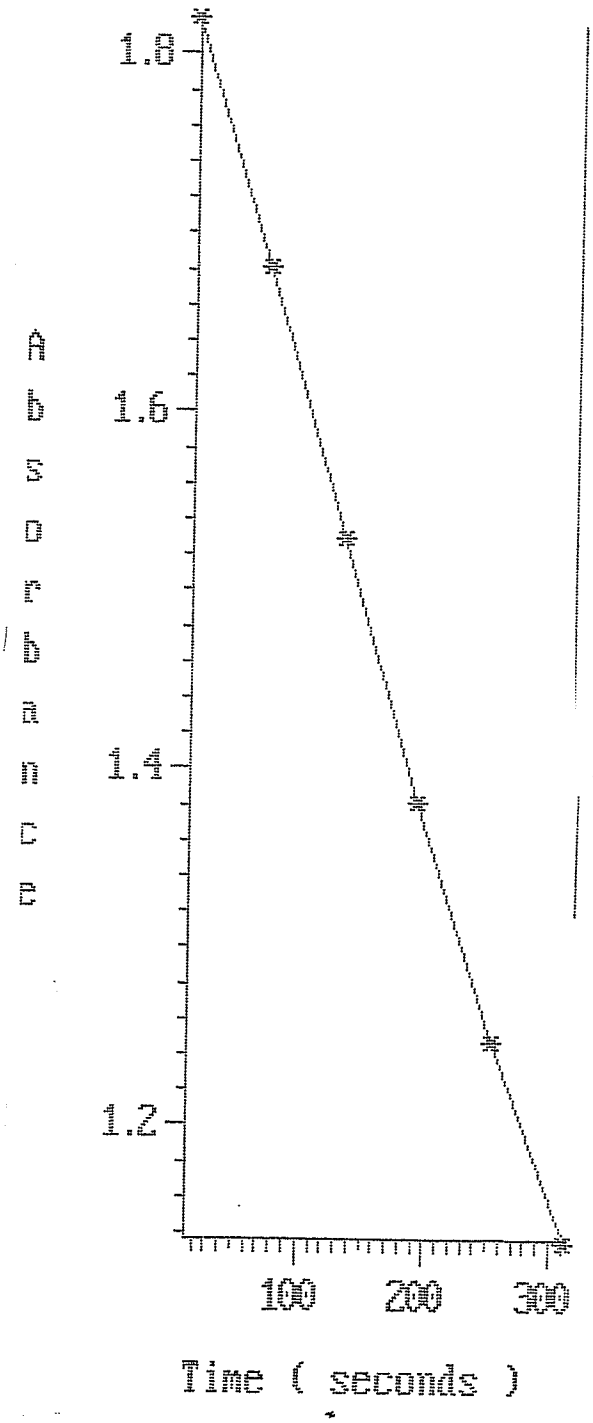
Rate Constant (/min)
2.58950e-001

* Abs/Min

** ABS/MIN/mg

Appendix F

OPTIMIZATION OF INTESTINAL GT_1 ACTIVITY



Initial Y:	1.821
Final Y:	1.135
Initial Time:	13.300
Final Time:	313.700
Rate:	-1.39140e-001 *
Factor:	-643.09
Calc:	8.94790e+001 **

LINEAR

Y-Intercept:	1.847
Corr. Coef.:	0.999
Std. Dev.:	0.009

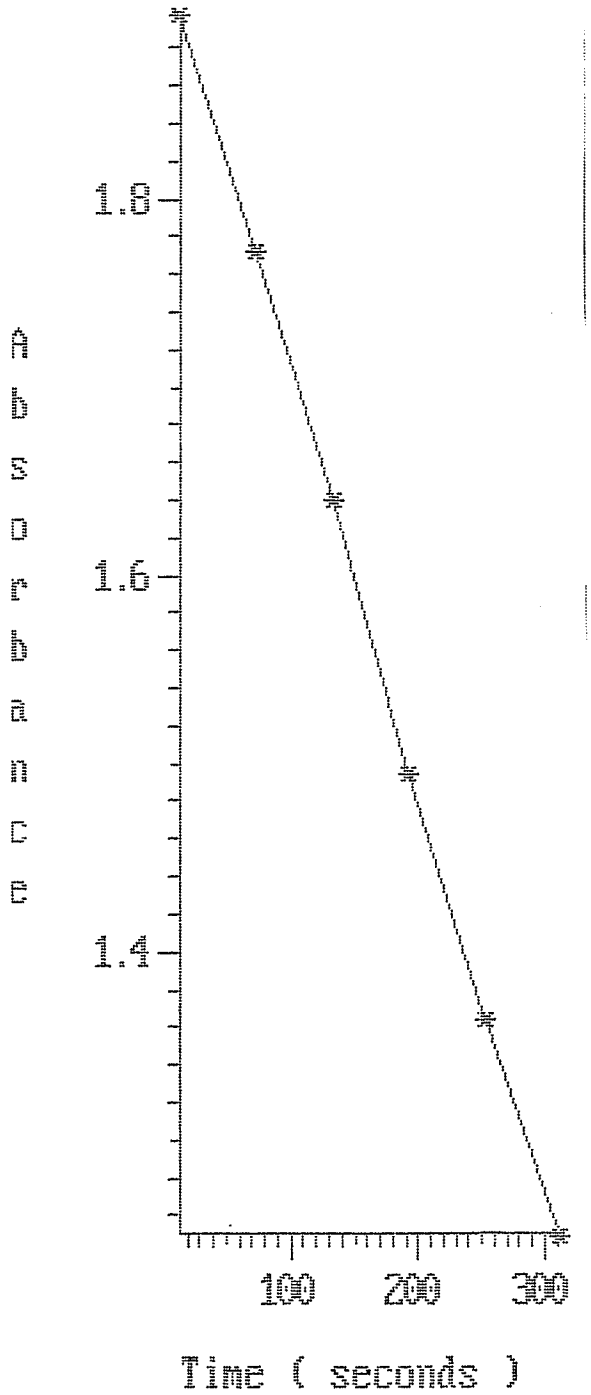
Rate Constant (/min)
2.58719e-001

* Abs/Min

** ABS/MIN/mg

Appendix G

OPTIMIZATION OF INTESTINAL GT_2 ACTIVITY



Initial Y: 1.900
Final Y: 1.250
Initial Time: 13.400
Final Time: 313.700

Rate: -1.31996e-001 *
Factor: -643.09
Calc: 8.48851e+001 **

LINEAR
Y-Intercept: 1.931
Corr. Coef.: 1.000
Std. Dev.: 0.006

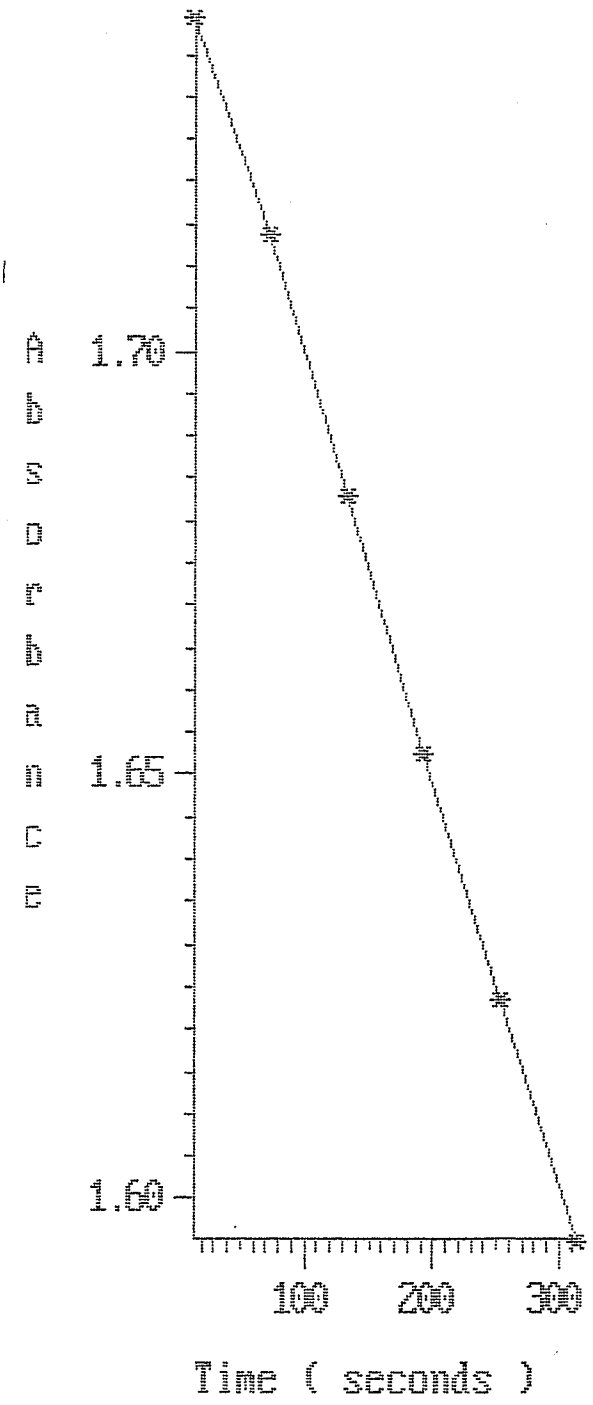
Rate Constant (/min)
2.44418e-001

* Abs/Min

** ABS/MIN

Appendix H

OPTIMIZATION OF KIDNEY GT_1 ACTIVITY



Initial Y:	1.740
Final Y:	1.595
Initial Time:	13.400
Final Time:	313.800

Rate: -2.92340e-002 *
Factor: -643.09
Calc: 1.88000e+001 **

LINEAR

Y-Intercept: 1.748
Corr. Coef.: 1.000
Std. Dev.: 0.001

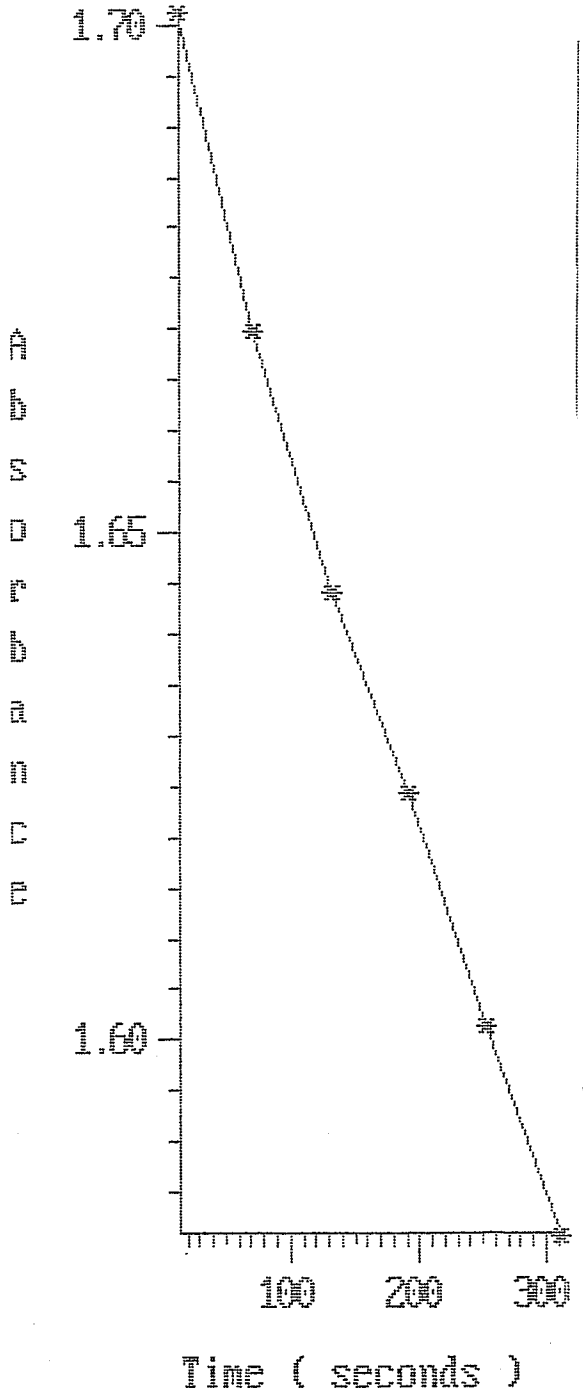
Rate Constant (/min)
2.33356e-001

* Abs/Min

** ABS/MIN/mg

Appendix I

OPTIMIZATION OF KIDNEY GT_2 ACTIVITY



Initial Y: 1.701
Final Y: 1.581
Initial Time: 13.300
Final Time: 313.800

Rate: -2.36295e-002 *
Factor: -643.09
Calc: 1.51958e+001 **

LINEAR
Y-Intercept: 1.702
Corr. Coef.: 0.997
Std. Dev.: 0.003

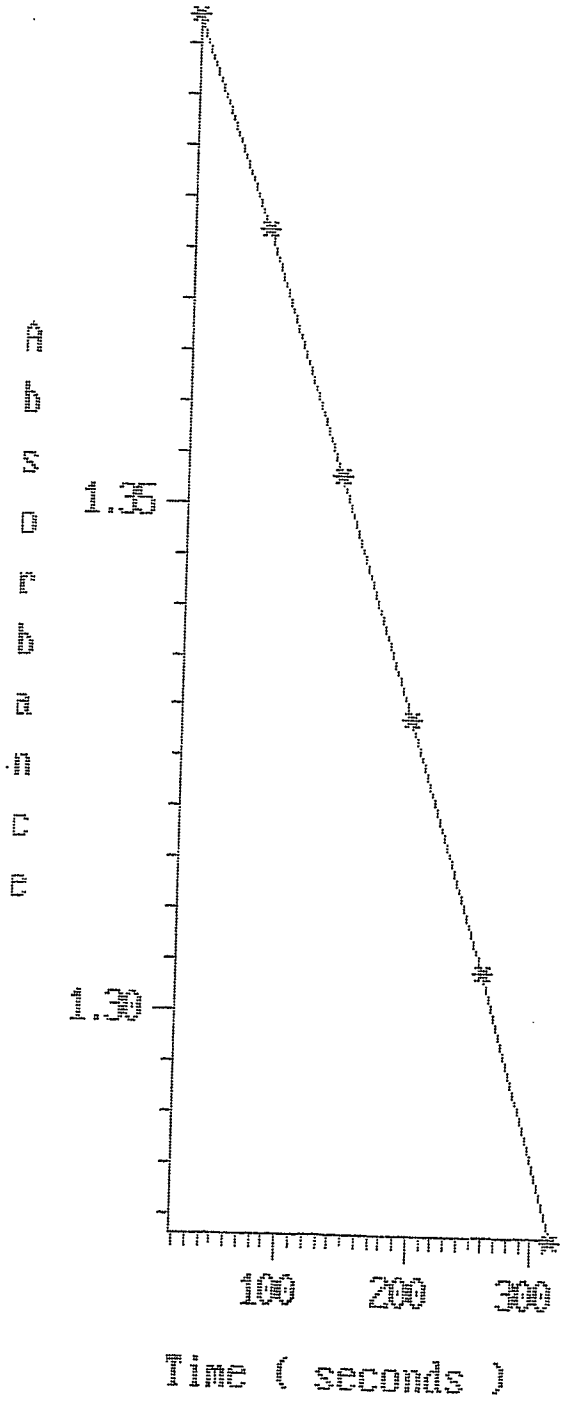
Rate Constant (/min)
4.28083e-001

* Abs/Min

** ABS/MIN/mg

Appendix J

OPTIMIZATION OF LIVER GT_1 ACTIVITY



Initial Y:	1.398
Final Y:	1.278
Initial Time:	16.500
Final Time:	317.000
Rate:	-2.40653e-002 *
Factor:	-643.09
Calc:	1.54761e+001 **

LINEAR

Y-Intercept:	1.407
Corr. Coef.:	0.999
Std. Dev.:	0.001

Rate Constant (/min)

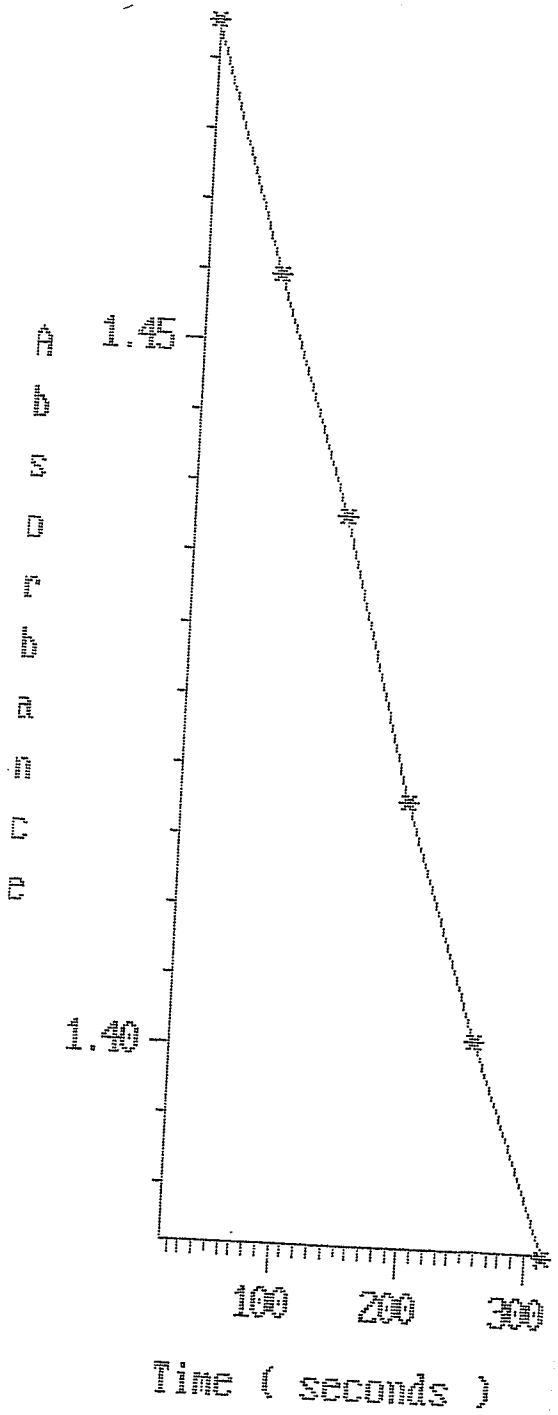
2.17628e-001

* Abs/Min

** ABS/MIN/mg

Appendix K

OPTIMIZATION OF LIVER GT_2 ACTIVITY



Initial Y: 1.473
Final Y: 1.386
Initial Time: 13.400
Final Time: 313.800

Rate: -1.75856e-002 *
Factor: -643.09
Calc: 1.13090e+001 **

LINEAR
Y-Intercept: 1.476
Corr. Coef.: 0.999
Std. Dev.: 0.001

Rate Constant (/min)
2.50471e-001

* Abs/Min

** ABS/MIN/mg

Appendix L

LIST OF ABBREVIATIONS

1)	BP	Benzo(a)pyrene
2)	BW	Body weight
3)	CCl_4	Carbontetrachloride
4)	C-P450	Cytochrome P-450
5)	GSH	Glutathione
6)	3MC	3-Methylcholanthrene
7)	MFO	Mixed function oxidase
7)	PB	Phenobarbital
8)	UDPGT	UDP-Glucuronyltransferase
9)	UDPGA	UDP-Glucuronic acid