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Modulation of Drug Metabolism by Food Restiction

Paul Kupen Su
University of Tennessee, Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Paul Kupen Su entitled "Modulation of Drug Metabolism by Food Restiction." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Human Ecology.

Dileep S. Sachan, Major Professor

We have read this dissertation and recommend its acceptance:

Frances Ann Draughon, Hugh O. Jaynes, Roy E. Beauchene

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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MODULATION OF DRUG METABOLISM BY FOOD RESTRICTION

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Paul Kupen Su

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ABSTRACT

The effects of the level and duration of feed restriction on the in vitro activities of hepatic drug metabolizing enzyme system were examined in male weanling Sprague Dawley rats fed ad libitum or feed restricted at 15%, 30% and 45% for a period of one to five weeks (Experiment I). Increasing levels and duration of feed restriction resulted in significant progressive increases in hepatic microsomal protein, cytochrome P-450 content and the in vitro activities of microsomal aniline hydroxylase. p-chloro-methly-aniline(PCMA)-N-demethylase and p-nitrophenol UDP-glucuronyl transferase activities were unaltered by the feed restriction while cytochrome c reductase activity was significantly decreased. In addition, the in vitro activities of the hepatic NADPH-generating enzymes were also significantly increased with the increasing levels and duration of feed restriction. It is concluded that the drug metabolizing enzymes did not necessarily change in concert with the cytochrome P-450 content and that prolonged feed restriction in healthy animals resulted in enhanced drug metabolizing capacity. This enhancement in drug metabolizing capacity was progressive with the increasing levels and duration of feed restrition. The greatest enhancement was observed when the animals were restricted at 45% for 4 or 5 weeks.

In two subsequent experiments, the in vivo metabolism of antipyrine and carbon tetrachloride toxicity were examined in rats feed restricted at 45% for four weeks. The feed restriction resulted in a

significant decrease in the blood half-life of antipyrine and increased morbidity due to carbon tetrachloride. Thus feed restriction resulted in increased in vivo metabolism of xenobiotics and 45% feed restriction for four weeks was sufficient to cause a significant increase. These results supported the changes observed in the in vitro activities of the drug metabolizing system.

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CHAPTER I

INTRODUCTION

Food restriction has become increasingly important not only for those with limited food supply in certain underdeveloped regions of the world but also for those in the affluent society for cosmetic as well as health reasons. The modification of diet has long been known to affect drug metabolism and the drug metabolizing enzyme (DME) system (1-16). This is of great significance because we are constantly exposed to a great amount of drugs and chemicals in our environment. Dietary modifications that can affect drug metabolism include a deficiency or lack of certain individual nutrients, or all nutrients as in starvation and food restriction.

Protein deficiency has been shown to decrease hepatic cytochrome P-450-dependent mixed-function oxidase (MFO) activity and increase the duration of the action of barbiturates by decreasing their rates of metabolism and tissue clearance in male rats (1). In a study of Asian lactovegetarians with low protein intakes, a reduced antipyrine clearance was reported (2). A low carbohydrate intake also has been reported to decrease hepatic cytochrome P-450 content and aminopyrine demethylase activity (3). Deficiency of polyunsaturated fatty acids has been shown to depress the capacity of the MFO system for induction (4) and a decrease in essential fatty acids in diets resulted in depressed basal MFO activities (5). A dietary deficiency of vitamin C in guinea pigs has been found to result in decreased MFO activities and their induction by organochlorine pesticides (6).

These are just some of the many studies on the deficiency of single nutrients.

A restriction of all nutrients such as encountered during fasting is also known to influence drug metabolism. The effects are dependent upon a number of factors such as species, sex and strain of the animals, and also the type of drug substrate used to determine the activity of the DME (7). The general concensus is that starvation impairs drug metabolism in the liver of male rats (8, 9, 10) and mice (11). Kato and Gillette (10) reported an impaired drug metabolism in starved male rats but not in starved female rats. These differences between sexes in drug metabolism were attributed to the influence of androgens on the hydroxylation and N-demethylation systems (12). There are numerous studies on the effects of starvation on drug metabolism but few on the effect of prolonged food restriction (semi-starvation) which is an issue of concern for many rich and poor people around the world.

Recently, it has been demonstrated that a 45-percent feed restriction for four weeks, unlike starvation, enhanced in vivo metabolism of hexobarbital and the in vitro activities of certain hepatic microsomal DME as well as the NADPH-generating enzymes in male rats (13, 14, 15). A 50-percent feed restriction for forty-nine days also increased the in vitro activities of biphenyl hydroxylase, 4-methylumbelliferone-glucuronyl transferase, and p-nitrobenzoate (PNBA)-reductase as well as glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD) and malic enzyme (ME) in the livers of male rats (16). These studies indicated that drug

metabolism and DME activities during prolonged food restriction is quite different from that caused by the deficiency of a single nutrient or by starvation.

The main objective of the study was to determine the effects of different levels and duration of feed restriction on drug metabolism by:

a. monitoring changes in the in vitro activities of the DME system in the liver;

b. determining the in vivo metabolism of antipyrine under defined conditions of feed restriction;

c. assessing the toxicity of carbon tetrachloride under defined conditions of feed restriction.

CHAPTER II

REVIEW OF LITERATURE

1. HEPATIC DRUG METABOLISM

It is well known that most drugs and environmental chemicals (xenobiotics) are metabolized primarily by the enzyme system located in the endoplasmic reticulum of the liver although the metabolism can also occur in the kidney, gastrointestinal tract, lung, gut flora, adrenal, brain, heart, muscle, skin, spleen and testes (17). The spherical membrane vesicles derived from the endoplasmic reticulum during homogenization of a tissue are called microsomes. These remain in the 10,000 x g supernatant and can be sedimented by differential centrifugation at 105,000 x g for 1 hr. The microsomes contain the majority of the enzymes responsible for the metabolism of xenobiotics (18).

In general, xenobiotics are metabolized by the soluble, mitochondrial and the microsomal fractions. This usually involves oxidation, reduction, hydrolysis and conjugation. The metabolism takes place in two phases. Phase I involves oxidative biotransformation of the drug, which introduces new functional groups (hydroxyl, carboxyl, amino and sulphydryl) into the drug molecules. Phase II reactions are synthetic reactions and involve conjugations of the parent compound or its metabolite with an endogenous substrate (glucuronic acid, glycine etc.) (17). The net result of these two phases is the conversion of a lipophilic molecule into a more polar

and hydrophilic molecule and thus readily excretable in urine breath or sweat.

The Phase I reactions metabolize xenobiotics as well as endogenous substrates such as steroids and fatty acids and are collectively termed "mixed function oxidases". These reactions are carried out by a membrane-bound enzyme system which involves at least two electron transport chains. One contains a hemoprotein, cytochrome P-450 and a flavoprotein, reduced nicotinamide adenine dinucleotide (NADPH) cytochrome c reductase. The other is comprised of the flavin enzyme NADH cytochrome b_5 reductase and a heme protein cytochrome b_5 (17,19,20). The main events of the cytochrome P-450 mediated drug oxidation scheme are illustrated in Figure 1 and briefly described as follows:

The ferric heme iron cytochrome P-450 ($P450.Fe^{3+}$) first reacts with the drug substrate (RH) to form a complex ($P450.Fe^{3+}.RH$). This complex then undergoes reduction to a ferrous heme iron cytochrome P-450 - substrate complex ($P450.Fe^{2+}.RH$) by the electron transfered from NADPH by cytochrome c reductase. The reduced complex ($P450.Fe^{2+}.RH$) then reacts with oxygen to form an oxycytochrome P-450 complex ($P450.Fe^{2+}.O_2.RH$) which may further undergo reduction to form a peroxide anion intermediate with a ferric heme iron cytochrome P-450. The second electron may originate from NADH via cytochrome b_5 reductase and cytochrome b_5. The intermediate then further dissociates to form the oxidized substrate, oxidized cytochrome P-450 and a molecule of water.

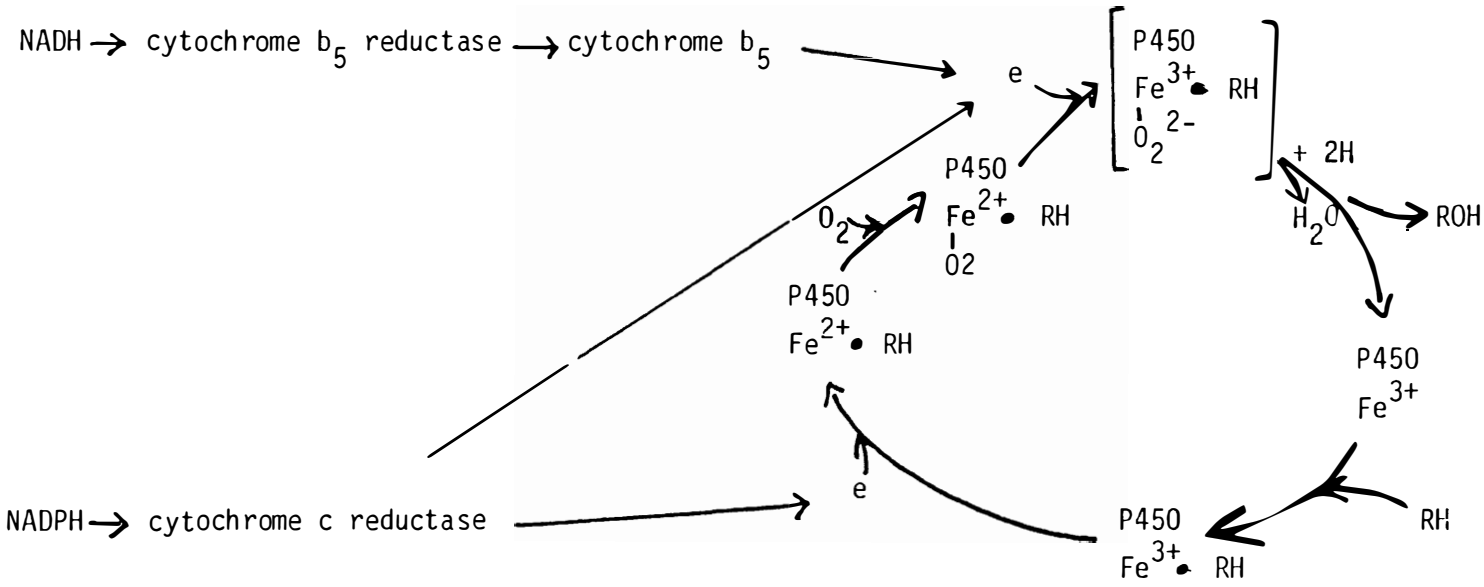
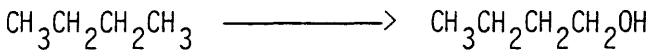


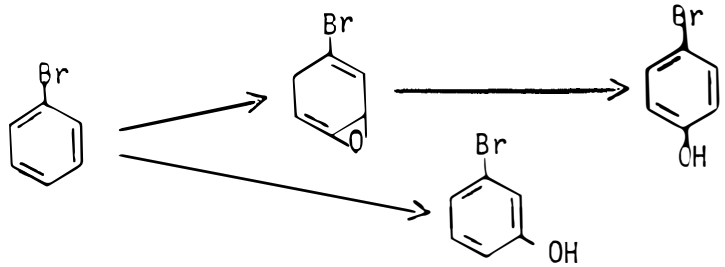
Figure 1. Proposed scheme of microsomal drug oxidation adapted from Wolf (19). RH = substrate e = electron; P450 Fe = heme iron of cytochrome P-450; O_2 = oxygen.

The oxidative reactions catalyzed by cytochrome P-450 are many and include aliphatic hydroxylation, aromatic hydroxylation, epoxidation, N-dealkylation, O-dealkylation, S-dealkylation, sulfoxidation, deamination as well as desulfuration (17,19,20). A brief summary of the main reactions encountered are as follows:

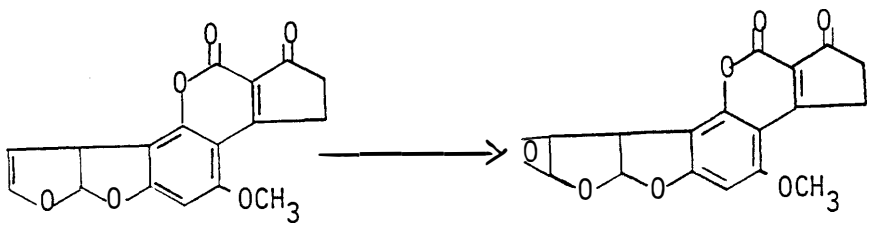
a) aliphatic hydroxylation--oxidation of aliphatic hydrocarbons producing alcohols. e.g. n-Butane.



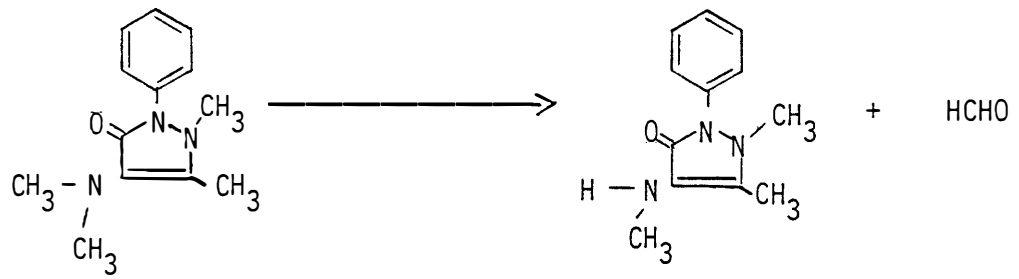
b) aromatic hydroxylation, epoxidation--oxidation of aromatic hydrocarbons predominantly yielding phenols. e.g. Bromobenzene.



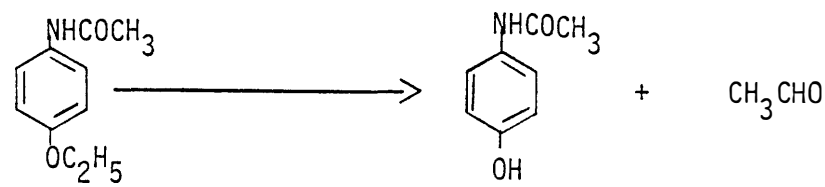
c) aliphatic epoxidation--oxidation of unsaturated hydrocarbons yielding epoxides. e.g. Aflatoxin B_1.



d) N-dealkylation--N-demethylation of secondary and tertiary amines yielding either primary or secondary amines together with an aldehyde. e.g. Aminopyrine.



e) O-dealkylation--O-demethylation or deethylation of ethers producing alcohols of phenols and formaldehyde or acetaldehyde respectively. e.g. Phenacetin.



f) S-dealkylation--S-demethylation of thioethers--not usually mediated by cytochrome P-450. e.g. 6-Methylthiopurine.

The Phase II reactions generally include synthetic reactions. It involves the coupling of the xenobiotics containing the hydroxyl, carboxyl, amino and sulfhydryl groups or their Phase I metabolites to an endogenous molecule such as glucuronic acid, sulfate, glutathione, glycine or other amino acids. This results in a more polar and excretable product (20). The major drug conjugation pathways are: glucuronic acid conjugation, sulfate conjugation, amide synthesis,

mercapturic acid synthesis, methylation, conjugation of substrate analogs and glucoside conjugation (18). In general, both oxidation and conjugation reactions may result in the termination of pharmacologic activity (inactivation) of a xenobiotic, however, there are many instances where the product is more toxic than the parent compound. Therefore, the same enzyme system that protects the organism from the harmful effects of certain toxic xenobiotics can also activate a biologically inert compound to a toxic product. The drug metabolizing system is influenced by a number of factors such as sex (10), species (30) age (31) and environmental factors. Among the latter, vegetables belonging to the Brassica genus such as Brussels sprouts, cabbage (22,23), charcoal broiled meats (24,25), cigarette smoke (26,27), soft wood bedding (28,29) and a variety of drugs metabolized by the mixed function oxidase system are also known to modify the drug metabolizing enzymes. Among these, the nutritional factors are of immediate interest here.

2. EFFECT OF DIETARY PROTEIN DEFICIENCY

Theoretically, a deficiency of dietary protein could result in a) catabolism of tissue protein, reducing the amount of amino acids available for protein synthesis which in turn reduces the amount of drug-metabolizing enzymes, and b) competition between the metabolism of drugs and the need of the tissues for the substrates used for conjugation of drugs (glucuronide, sulphate, glycine, etc.) as nutrients (32). In 1973, Mgbodile et al. (33) demonstrated that the consumption of protein-deficient diets resulted in lower cell

numbers in the liver as reflected by decreased DNA content and less microsomal protein per gram of liver. Decreased activities of hepatic microsomal enzymes specific for pentobarbital, strychnine, zoxazolamine and aminopyrine were observed when weanling rats were fed either a protein-deficient diet (5% and 10% protein) for two weeks or a protein-free diet for four days (34). Hayes and Campbell (35), in feeding rats 5% and 20% protein diets for 14 days, reported that the hepatic cytochrome P-450 content (0.16 $\pm$ 0.03 vs 0.41 $\pm$ 0.07 nmoles/mg protein), ethyl morphine N-demethylase, (400 $\pm$ 48 vs 1200 $\pm$ 70 nmoles HCHO produced/hr/mg protein) and aniline hydroxylase (8.6 $\pm$ 1.2 vs 23.8 $\pm$ 1.8 nmoles p-aminophenol/hr/mg protein) activities were to be decreased in rats fed the low protein diet. They further demonstrated that the protein deficiency also impaired the inducibility of these enzymes. Their observations are in agreement with those of Marshall and McLean (36) who reported that the activities of mixed function oxidase system were related to dietary protein intake. Recently, Kawano and Hiraga (37), in feeding rats a protein-free diet for four to 67 days, reported that the decrease in the drug-metabolizing enzymes were biphasic (decreasing rapidly during the first three-week phase and then at a much slower rate) and that the inducibility of the enzymes was diminished with increased severity of protein depletion. The activity of glucuronyltransferase, however, was reported to be increased in protein-deficient rats (38). The compounds, such as carbon tetrachloride (39) and heptachlor (40), which are activated by microsomal metabolism, were found to be less toxic in rats fed diets low in proteins. The effect of protein

deficiency, therefore, does not necessarily translate into increased toxic effects of all foreign compounds.

3. EFFECT OF DIETARY LIPIDS DEFICIENCY

Since most of the drug-metabolizing enzymes are located in membrane structures, the metabolic fate of xenobiotics is also influenced by the permeability of the subcellular membranes to these compounds. The requirement of lipids for proper function of the enzymes bound to those membranes is well established (4,32,41,42). Kamataki and Kitagawa (42) reported a decrease in hepatic drug-metabolizing enzymes when the microsomes were subjected to peroxidation. Strobel et al. (32) reported that phosphatidylcholine appears to be essential for the NADPH-dependent reduction of cytochrome P-450 and drug hydroxylation. Di Augustine and Fouts (41) further suggested that fatty acids must be incorporated into the membrane phospholipids to be functional since the addition of unsaturated fatty acids to an incubation mixture resulted in inhibition of the drug metabolizing enzymes.

The role of the degree of saturation of dietary lipids has also been extensively studied. The effect of saturated fatty acids on drug metabolism was dependent on the absolute amount of fat rather than the chain length. An increase in the saturated fatty acid content in the diet from 15% to 35% resulted in a substantial increase in aniline hydroxylase activity (43). However, unsaturated fatty acids are found to be more important in promoting the mixed function oxidase activity (44). This is in agreement with the

findings of Norred and Wade (45), who later reported that a diet containing unsaturated fatty acids (3% corn oil) better supported the phenobarbital induction of hexobarbital oxidase and aniline hydroxylase than a similar diet containing saturated fatty acids (3% coconut oil). In 1978, Hietanen et al. (46) suggested that dietary cholesterol may be essential in maintaining the activities of certain drug-metabolizing enzymes such as p-nitroanisole O-demethylase, ethoxycoumarin O-deethylase, and UDP-glucuronyltransferase, but not cytochrome P-450 content. In a later study, they reported that the cytochrome P-450 content, diphenyloxazole hydroxylase and ethoxycoumarin O-deethylase activities were all increased about twofold in the livers of their rats that were fed a 2% cholesterol diet, as compared to those fed a cholesterol-free diet (47). The hepatic epoxide hydrolase activity was increased about threefold, while the hepatic UDP-glucuronyltransferase activity was increased 1.5-fold in the rats fed the 2% cholesterol diet. The cholesterol-fed rats exhibited greater phenobarbital inducibility of the drug-metabolizing enzymes (47). They concluded that 2% dietary cholesterol for four weeks has potent effects on the structure of endoplasmic reticulum in the liver, and is able to alter the response of drug-metabolizing enzymes in the membrane. Thus, a dietary lipid deficiency, especially in the form of unsaturated fatty acids, can be expected to result in a decrease in the metabolism of drugs and foreign compounds. This premise is in agreement with the findings of Norred and Wade (48), who reported that rats fed a synthetic fat-free

diet had a significantly lower cytochrome P-450 content, when compared with the controls.

Therefore, certain dietary lipids are required for the normal synthesis of the mixed-function oxidase components and for the phenobarbitone induction of microsomal cytochrome P-450 and hydroxylating enzymes (4, 48).

4. EFFECT OF DIETARY CARBOHYDRATE DEFICIENCY

Investigations on the effects of different dietary protein levels on drug metabolism, were done essentially by replacing the protein content of the diet by carbohydrates. Thus, the high-protein diets were essentially carbohydrate-deficient diets. This led Nakajima et al. (49) to believe that the effects of protein in drug metabolism were actually that of carbohydrates. In their study, the effects of protein, fat and carbohydrate were examined by utilizing a series of diets in which the content of one of the nutrients is varied at the expense of one of the other two nutrients without changing the total caloric content. They demonstrated that feeding a sucrose-free diet for one day resulted in increased metabolic rates of hydrocarbons comparable to that found in rats that had been completely deprived of food for one day. Diets with decreased sucrose content resulted in increased metabolism of hydrocarbons, whereas diets with varied protein or fat content (the sucrose content being constant) did not result in significant changes in the metabolism of hydrocarbons examined. Therefore, they concluded that the reported effects of protein, fat and one-day fasting were

actually the effects of carbohydrate (49). The effects of high and low dietary carbohydrate on hepatic drug metabolism was investigated by Sonawane et al. (3) utilizing starch and sucrose. They demonstrated that a low-starch or low-sucrose diet for four weeks resulted in an increased hepatic microsomal protein concentration while the cytochrome P-450 content and aminopyrine demethylase activity were decreased. The cytochrome P-450 content in the control animals when compared to that in the low-starch and low-sucrose animals were 0.62 $\pm$ 0.02 vs 0.48 $\pm$ 0.025 and 0.50 $\pm$ 0.021 nmoles/mg protein, respectively. The aminopyrine demethylase activity in the control animals was 39.6 $\pm$ 0.04 whereas the activities in the low-starch and low-sucrose animals were 21.0 $\pm$ 0.60 and 19.0 $\pm$ 0.74 nmoles/mg protein/20 min incubation, respectively. The p-nitrophenol-UDP-glucuronyltransferase activity on the other hand was significantly increased from the basal rate (20.2 $\pm$ 0.60 , 32.2 $\pm$ 1.66 and 32.9 $\pm$ 1.63 nmoles/mg protein/5 min incubation, for the control, low-starch and low-sucrose groups respectively) (3).

5. EFFECT OF VITAMIN DEFICIENCY

Fat-soluble vitamins

Rats fed a vitamin A-deficient diet were reported to have significantly lower hepatic drug hydroxylation and demethylation activities and cytochrome P-450 content (50). Siddik et al. (51) reported that weanling rats fed a vitamin A deficient diet for eight weeks had lower hepatic cytochrome P-450 contents and N-methyl-p-chloroaniline N-demethylase activity, however, the

activities of NADPH-cytochrome C reductase, p-nitrobenzoic acid nitroreductase and biphenyl hydroxylase were not affected. Their findings are supported by the recent observations of Dogra et al. (52) who reported that vitamin A deficiency for five to six weeks resulted in decreased cytochrome P-450 content (0.74 $\pm$ 0.10 vs 0.09 nmoles/mg protein), benzo(a)pyrene hydroxylase (146.00 $\pm$ 18.93 vs 90.00 $\pm$ 22.76 picomoles/mg protein/min) and UDP-glucuronyltransferase (2.67 $\pm$ 0.47 vs 2.40 $\pm$ 0.27 nmoles of product formed/min/mg protein) activities (52). The molecular role of vitamin A in drug metabolism has not yet been elucidated (51) and it has been suggested that vitamin A may have a structural role in membranes (53), thus affecting drug metabolism. Vitamin E deficiency has been also reported to result in decreased drug metabolism (54). Not much information was available on the effects of diets deficient in the other fat soluble vitamins (D and K) on drug metabolism.

Water Soluble Vitamins

Wade et al. (55), in feeding rats 0, 0.03, 0.11, 0.20, 0.49, 1.0, 2.9, 7.0, 15.0, 30, 50, 100, 500 and 2000 g thiamine HCl daily, reported that the in vitro metabolism of heptachlor and aniline decreased as the level of thiamine intake increased. In vitro hexobarbital metabolism, on the other hand, was unaffected by the different levels of thiamine intake. Their findings were consistent with observations by Grosse and Wade (56), who reported a significantly lower drug metabolism in rats receiving a high-thiamine diet. They suggested that the higher drug metabolism rates found in

rats fed the lower thiamine diet may be due in part to the effects of chronic feed restriction since the animals on the low-thiamine diets consumed less feed and gained significantly less weight than those fed by the high-thiamine diet (55).

Since the hepatic microsomal electron transport chain involves the NADPH-specific flavoprotein (NADPH-cytochrome c reductase), Catz et al. (57) investigated the effects of riboflavin deficiency in mice and reported an initial decrease in hexobarbital sleeping time after ten days on the deficient diet, but a threefold increase in the sleeping time one week later when compared to the controls. However, they could not correlate their in vivo observations with the in vitro rates of metabolism of the type I substrates, namely aminopyrine and hexobarbital, which remained unaffected. The cytochrome P-450 content and aniline hydroxylase activity were increased while benzopyrene oxidation was decreased (57).

Numerous studies in vivo and in vitro have demonstrated that the metabolism of drugs by the mixed-function oxidase system is decreased in ascorbic acid deficiency. These studies have revealed that an ascorbic acid deficiency resulted in decreased cytochrome P-450 content, cytochrome b_5 level, cytochrome P-450 reductase, aniline hydroxylase and benzphetamine N-demethylase activities (58-64) however, cytochrome C reductase activity, was unaffected (63). The biochemical basis for the role of ascorbic acid in drug metabolism has yet to be elucidated, although recent studies by Turnbull and Omaye (62) suggested a role in the assembly of the

active form of cytochrome P-450, including apocytochrome synthesis and the incorporation of Fe^{2+} in the heme moity of cytochrome P-450.

6. EFFECT OF STARVATION

Starvation has long been known to influence drug metabolism, and Gram et al. (65) pointed out that different microsomal enzymes responded differently to the effects of starvation. Starvation for one to three days has been shown to have no effect on hepatic microsomal protein concentration (10,66-68), cytochrome P-450 content (8,66-69) and cytochrome c reductase activity (69), although Pessayre et al. (69) reported an increase in the microsomal protein concentration after a 42-hour fast. Impaired metabolism of aminopyrine and hexobarbital was observed in starved male rats by Kato and Gillette (10). Certain drug metabolizing enzymes such as ethylmorphine demethylase (68, 69) and benzopyrene hydroxylase (69) were also reported to be decreased, while aniline hydroxylase (237 $\pm$ 16 vs 388 $\pm$ 25 nmoles/g liver/30 min incubation) (10), nitroso-dimethylamine demethylase (70) and p-nitroanisole demethylase (69) were increased following a period of fasting. Glucuronyl transferase activity has been reported to increase with fasting (66, 71). Duvaldestin et al. (71) in their study of UDP-glucuronyl transferase using bilirubin, phenolpthalein and p-nitrophenol as substrates found that only the bilirubin UDP-glucuronyltransferase activity was increased after a 3-day fast. Previously, Bock et al. (72) and Zakim et al. (73) had stated that the glucuronyltransferases were a heterogeneous group of closely related enzymes with different

specific substrates and the results of Duvaldestin et al. (71) lend further support to this argument, while demonstrating the variability of the response of certain enzymes to fasting.

When interpreting the in vitro activities of these enzymes to fasting, one often has to take into account the change in liver size. Fasting has been demonstrated to result in a decreased liver weight (65) as well as a decreased liver weight/body weight ratio (69). This decrease in liver size translated into lesser amount of total enzyme activity as demonstrated by Marselos and Laitinen (8) and Duvaldestin et al. (71), although increased in vitro activities of various hepatic microsomal drug metabolizing enzymes calculated per whole liver have been reported in rats fasted for three days (67). The overall effect of fasting in general is a decrease in drug metabolism (10,74,75).

In severe starvation (up to four days), Carlson (76) reported an induction of the drug metabolizing enzyme system and suggested that the severe starvation probably mobilized the xenobiotics in the fat stores and thus induced the drug metabolizing system. Presumably, polychlorinated biphenyls are stored in body fat for considerable periods of time (77).

7. EFFECT OF FEED RESTRICTION

Studies examining the effects of prolonged partial food restriction on the metabolism of drugs and environmental xenobiotics are rather limited. Sachan (14) reported that feed restriction at 45% for 28 days resulted in a significant decrease in hexobarbital

sleeping time, and increased hepatic cytochrome P-450 content and activities of drug metabolizing enzymes. NADPH-generating enzymes were also reported to be increased by the 45% feed restriction. These findings were supported by the observations of Sachan and Das (16) where increases in drug metabolizing enzymes as well as the NADPH-generating enzymes were reported when rats were feed restricted at 50% for a period of 49 days. These studies indicate that prolonged feed restriction results in increased drug metabolism unlike that observed in starvation or single nutrient deficiencies. Since only one level of feed restriction was examined in each of these studies, it was not possible to determine at what degree of feed restriction these change would occur. We examined this question in a recent study where 15, 30 and 45% feed restriction were imposed for four weeks and found that 45% feed restriction was most effective in altering certain drug metabolizing enzymes but not others (15).

None of these studies provided information about the duration of restriction necessary at any level of feed restriction at which changes in DME would be expected to be seen first. It was argued that perhaps this concern may be best resolved by simultaneously examining the effects of degree and duration of feed restriction. Therefore, in the current study, the effect of both the level as well as the duration of feed restriction on drug metabolism was undertaken to examine these issues.

CHAPTER III

PROCEDURE

1. EXPERIMENT I

Experimental Design

Male weanling Sprague-Dawley rats (Harlan Sprague-Dawley Inc., Indianapolis, Indiana) weighing approximately 60 g were divided into a control group of 20 animals and 15 treatment groups of five animals each. The animals were allowed to aclimatize to the laboratory conditions for one week before the onset of the experiment. The beginning dates of the experiment for each block of animals were staggered so that only a maximum of ten animals will have completed the duration of experiment on each termination day. All animals were of the same age when they started on the experiment and they were all terminated at the end of the five week experimental period. The animals were individually housed in 7" x 10" x 7" wire mesh stainless steel cages and distilled water was made available ad libitum. The animals in group 1 (control group) were fed a semi-synthetic normal diet (Table 1) ad libitum. Groups 2 through 6, 7 through 11, and 12 through 16 were given the same semi-synthetic diet at a level equal to 85%, 70% and 55%, respectively, of the average amount consumed by the ad libitum group on the preceding day. The duration of restriction for each level of restriction was from one to five weeks depending on the treatment group (Table 2). All animals were sacrificed at the end of a five-week experimental

TABLE 1

COMPOSITION OF THE EXPERIMENTAL DIET

Ingredient	Composition (%)
Casein	20.0
Sucrose	29.0
Cornstarch	31.0
Alphacel	6.0
Crisco	6.0
Wesson Oil	2.0
Vitamin mix[1]	2.0
Salt mix[2] (with Se[3] & Zn[4])	4.0

[1]Vitamin diet fortification mixture, ICN Pharmaceuticals Inc., Life Sciences Group, 26201 Miles Road, Cleveland, Ohio 44128. Composition per kg of mixture: (in g) vitamin A concentrate (200,000 IU/g), 4.5; vitamin D concentrate (400,000 IU/g) 0.25; -tocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; menadione, 2.25; p-amino benzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine hydrochloride, 1.0; thiamin hydrochloride, 1.0; calcium pantothenate, 3.0; (in mg) biotin, 20; folic acid, 90; vitamin B-12, 1.35.

[2]Hubbell, Mendel and Wakeman salt mixture, ICN Pharmaceuticals Inc., Life Sciences Group, 26201 Miles Road, Cleveland, Ohio 44128. Composition of mixture: calcium carbonate 54.3%; magnesium carbonate 2.5%; magnesium sulfate ($7H_2O$) 1.6%; sodium chloride 6.9%, potassium chloride 11.2%; potassium phosphate (monobasic) 21.2%; ferric phosphate 2.05%; potassium iodide 0.008%; manganese sulfate (H_2O) 0.035%; sodium fluoride 0.10%; aluminum potassium sulfate 0.017%; copper sulfate ($5H_2O$) 0.09%.

[3]$NaSeO_4$ @ 0.0003% of salt mix.

[4]$ZnCO_3$ @ 0.06% of salt mix.

TABLE 2

EXPERIMENTAL DESIGN FOR EXPERIMENT I

Group No.	No. of Animals	Level of Feed Restriciton	Duration of Feed Restriction	Onset of Feed Restriction[1] During the Experimental Period (week) 1	2	3	4	5
1	20	0%	0					
2	5	15%	1 week					-------->
3	5	15%	2 weeks				---------->	
4	5	15%	3 weeks			---------->		
5	5	15%	4 weeks		---------->			
6	5	15%	5 weeks	---------->				
7	5	30%	1 week					-------->
8	5	30%	2 weeks				---------->	
9	5	30%	3 weeks			---------->		
10	5	30%	4 weeks		---------->			
11	5	30%	5 weeks	---------->				
12	5	45%	1 week					-------->
13	5	45%	2 weeks				---------->	
14	5	45%	3 weeks			---------->		
15	5	45%	4 weeks		---------->			
16	5	45%	5 weeks	---------->				

[1]All animals were fed ad libitum until the onset of their respective restriction periods.

period. All animals were fed ad libitum until the onset of their respective restriction periods. For example, if the restriction was for a period of two weeks, the animals in that group would be fed ad libitum for the first three weeks and then feed restricted during the last two weeks of the experimental period. Thus the animals also served as their own controls during the first period of the experiment. Due to the large number of animals involved, and the fact that only a maximum of ten animals could be handled and processed properly at a given time, an incomplete block design was employed. The designation of the animals to the block are described as follows:

Attempts were made to achieve approximate balance in the incomplete block design utilizing procedures available in general linear models (GLM) of Statistical Analysis Systems (SAS) 1979 (78). Animals were randomly assigned (on paper) to each block manually and then a computer analysis of the design was run using a pseudo-response variable for the purpose of looking at relative standard errors among the least square means of the treatment group.

While inspecting the standard error and looking at the effective number of animals per treatment, if the standard errors were found to be greatly different from each other, the animals were reassigned within the block and the design reanalyzed. The process was repeated until all the standard errors were within a close range of each other. This was to ensure that 1) all treatment groups were estimable and 2) each treatment mean was estimated with approximately the same precision.

Sample Collection and Analysis

At the end of the five-week experimental period, the animals in Experiment I were sacrificed under sodium Brevital (Eli Lilly and Co., Indianapolis, Indiana) anesthesia. Blood was collected in heparinized tubes (approximately 0.2 ml of 5 mg/ml heparin solution per 7 ml of whole blood) by heart puncture and plasma was obtained by centrifugation at 1500 x g at 4°C for ten minutes in a refrigerated centrifuge (Model J-6B, Beckman Instruments, Palo Alto, CA) and stored in a freezer at -60°C. The viscera was exposed and a portion of the right lateral lobe of the liver was freeze-clamped with a pair of flat thongs with aluminum jaws (made by riveting 6 x 2 x 1 cm^3 aluminum blocks to the jaws) previously chilled in liquid nitrogen. The frozen piece of liver was weighed quickly and then stored in a freezer at -60°C until analyzed. Various organs such as heart, kidney, skeletal muscle and small intestine were excised quickly, frozen immediately in liquid nitrogen and stored in a freezer at -60°C for possible future analyses. The rest of the liver was excised, weighed, minced with a pair of surgical scissors while on ice. A small portion of the minced liver was stored at -60°C for future analyses and the remaining portion used for subcellular fractionation, immediately.

Subcellular Fractionation

The freshly minced liver was homogenized (3 strokes) in three volumes of buffer (1.15% KCl--0.01 M KPO_4 phosphate, pH 7.4) in a glass homogenizer with a teflon pestle driven by a low-speed motor

while on ice. The homogenizer was then rinsed with the buffer and more buffer was added to the homogenate to obtain a concentration of approximately 12% (w/v). The homogenate was centrifuged at 10,000 x g for 10 minutes at 4°C. A portion of the supernatant (SN) was saved and frozen at -60°C for further analyses while the remainder of the 10,000 x g SN was centrifuged at 100,000 x g for 1 hr. at 4°C (Model L5-50 Ultracentrifuge, Beckman Instruments, Inc., Palo Alto, CA). An aliquot of the 100,000 x g SN was then saved and frozen at -60°C until analyzed while the pellet (microsomes) was resuspended in a glycerol:phosphate buffer (1:1-glycerol-0.15 M KPO_4, pH 7.8) without washing. The volume of the homogenate, various supernatants, and the microsomal suspension were recorded at each stage to allow for calculations of dilution factors.

The microsomes were used to determine protein content (79) cytochrome P-450 content (21), and NADPH-cytochrome c reductase activity (80) immediately after preparation (usually late afternoon). The remainder of the microsomal suspension was frozen at -60°C until aliquots were used to determine the in vitro activities of aniline hydroxylase (81), p-chloro-N-methylaniline demethylase (82) and UDP-glucuronyl transferase (83) on subsequent days (usually within a week) with the exception of UDP-glucuronyl transferase. The 100,000 x g SN were used to determine protein content (84), glucose 6-phosphate dehydrogenase (85), 6-phosphogluconate dehydrogenase (85) and malic enzyme (86) activities.

Assay Procedure

Before analyzing experimental samples, each assay procedure was subjected to quality control, i.e. substrate and time courses were established for the linear range of an assay. Microsomal protein content, Cytochrome P-450 content and NADPH-cytochrome c reductase activity were determined within 24 hours of termination while the 100,000x g SN protein and NADPH-generating enzymes were determined within 48 hours of termination. Aniline hydroxylase and PCMA-N-demethylase activities were determined within a week of termination while p-nitrophenol-UDP-glucuronyl transferase activity was determined within a year.

Protein Determination

Microsmal protein was determined by the procedure of Lowry et al. (79)

Reagents

1. Sodium carbonate (Na_2CO_3) in 0.1 N sodium hydroxide (NaOH) 2% (w/v): 6 g of Na_2CO_3 was dissolved in 0.1 N NaOH and brought up to a total volume of 300 ml.

2. (0.1 N) NaOH: 2 g NaOH was dissolved in glass distilled water (GDW) and brought up to a total volume of 500 ml.

3. Cupric sulfate ($CuSO_4.5H_2O$), 1% (w/v): 0.5 g $CuSO_4.5H_2O$ was dissolved in GDW and the total volume brought up to 50 ml.

4. Sodium potassium tartrate ($NaKC_4H_4O_6.4H_2O$), 2% (w/v): 1 g of $NaKC_4H_4O_6.4H_2O$ was dissolved in GDW and brought up to a total volume of 50 ml.

5. Diluted folin reagent: Folin & Ciocalteu's phenol reagent (Fisher Scientific Co., Fair Lawn, NJ) was diluted 1:1 (v/v) with GDW and stored in an amber bottle at $4^{o}C$.

6. Alkaline copper reagent: 0.5 ml of the 2% $NaKC_4H_4O_6.4H_2O$ and 0.5 ml of the 1% $CuSO_4.5H_2O$ solutions were added to 50 ml of the 2% Na_2CO_3 in 0.1 N NaOH solution. The solution was mixed thoroughly to avoid precipitation of $Cu(OH)_2$. This solution was prepared just prior to the first run and discarded at the end of the day.

7. Bovine serum albumin stock standard: 10 g of crystalline bovine serum albumin (ICN Pharmaceuticals, Inc., Life Sciences Group, Cleveland, OH) was dissolved in 100 ml of 0.9% saline. A working standard was made by diluting the stock standard to obtain a concentration of exactly 0.5 mg/ml and kept at $0^{o}C$.

Procedure. The microsomal suspension was diluted tenfold with GDW and 0.01 ml aliquots of the diluted microsomal suspension were used for the assay. Two ml of the alkaline copper reagent was added to the 0.01 ml aliquots of diluted microsomal suspension and the total volume was brought up to 3 ml with GDW in a test tube. The mixture was vortexed and allowed to stand at room temperature for at least ten minutes before 0.2 ml of the diluted folin reagent was added and followed immediately by vigorous mixing. The mixture was allowed to stand at room temperature for 30 minutes and the absorbance at 750 nm was measured (Spectronic 21, Bausch & Lomb Inc., Rochester, NY) against a reagent blank. The reagent blank was prepared by adding GDW in the place of the diluted microsomal suspension. A standard curve was determined by using seven

concentrations (5, 15, 30, 50, 60 and 75 μg) of the working serum albumin standard in place of the sample. A standard curve is shown in Figure 2. The calculations were as follows:

$$\text{Protein } (\mu g/ml) = AU/AS \times CS \times DF$$

where:

AU = absorbance of unknown

AS = absorbance of standard

CS = concentration of standard

DF = dilution factor

100,000 x g SN Protein Determination.

Protein content in the 100,000 x g SN was determined by the biuret technique as described by Gornall et al. (84).

Reagents.

1. Biuret reagent: 90 g $NaKC_4H_4O_6.4H_2O$ was dissolved in about 400 ml of 0.2 N NaOH solution. Ten g of $CuSO_4.5H_2O$ and 10 g potassium iodide (KI) were added to it and the total volume was made up to 2 liters with 0.2 N NaOH.

2. Standard bovine serum albumin: 10 g of crystalline bovine serum (ICN Pharmaceuticals, Inc., Life Sciences Group, Cleveland, OH) was dissolved in 100 ml of 0.9% saline. A working standard was made by diluting the stock solution to obtain a concentration of 40 mg/ml.

Procedure. A 0.4 ml aliquot of the 100,000 x g SN was added to a test tube, and the volume was made up to 1 ml with GDW. Five ml of biuret reagent was added and the solution was vortexed. After

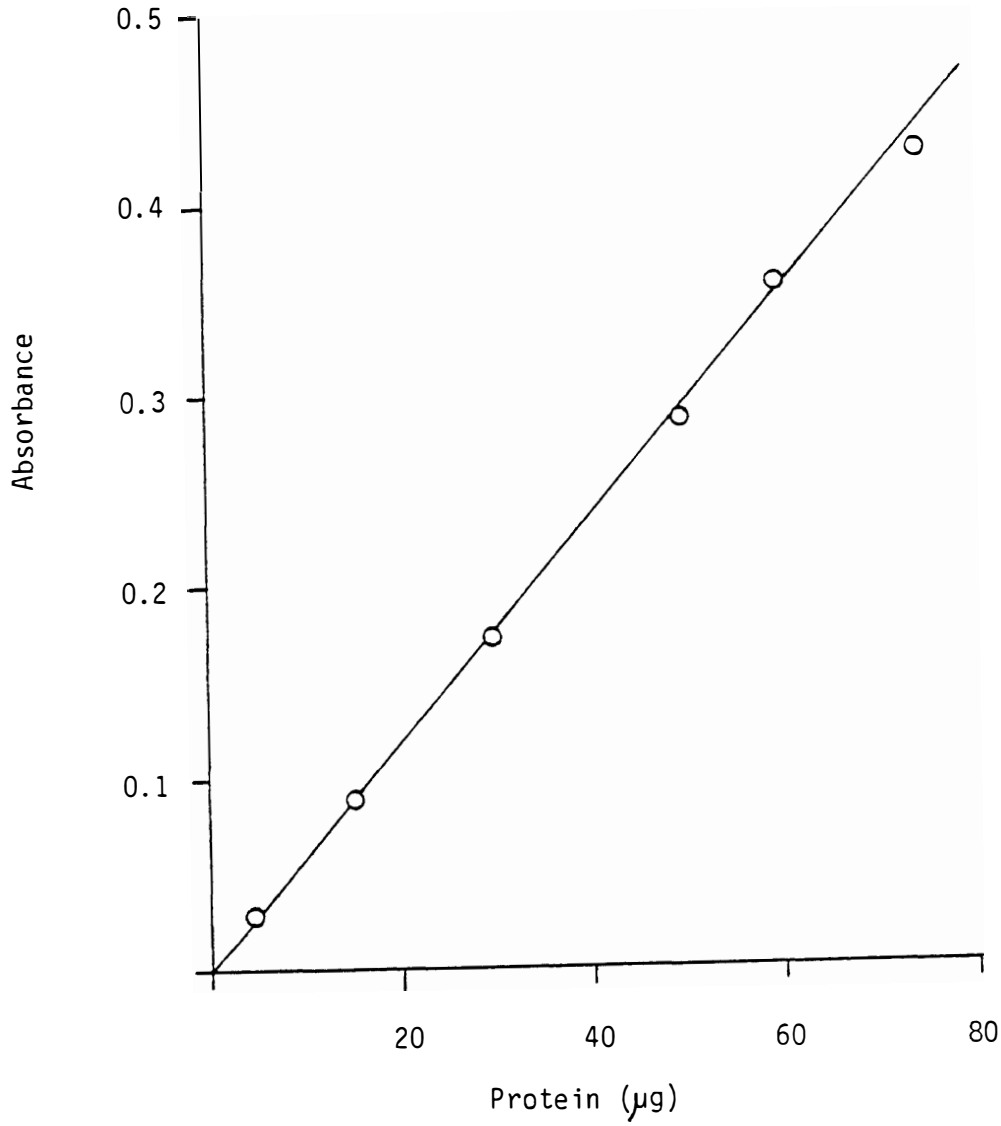


Figure 2. Standard curve for microsomal protein obtained with BSA at 750 nm.

standing for 30 minutes in room temperature, the absorbance was measured at 540 nm against a reagent blank. The reagent blank was prepared by adding GDW in place of the 100,000 x g SN. A standard curve was determined by using five concentrations (0.04, 2.0, 4.0, 8.0 and 16.0 mg) of the working serum albumin standard in place of the sample. A standard curve is shown in Figure 3. The calculations were as follows:

$$\text{Protein (mg/ml)} = AU/AS \times CS \times DF$$

where:

AU = absorbance of unknown

AS = absorbance of standard

CS = concentration of standard

DF = dilution factor

Cytochrome P-450 Content

The cytochrome P-450 content in the microsomes was determined by the procedure of Omura and Sato (21). The principle in this procedure is based on the carbon monoxide binding of the sodium dithionite reduced cytochrome P-450 hemoprotein which has an intense absorbance peak at 450 nm.

Reagents.

1. 0.6 M dipotassium phosphate: 52.25 g of K_2HPO_4 was dissolved in GDW and brought up to a total volume of 500 ml.

2. 0.6 M monopotassium phosphate: 20.41 g of KH_2PO_4 was dissolved in GDW and brought up to a total volume of 250 ml.

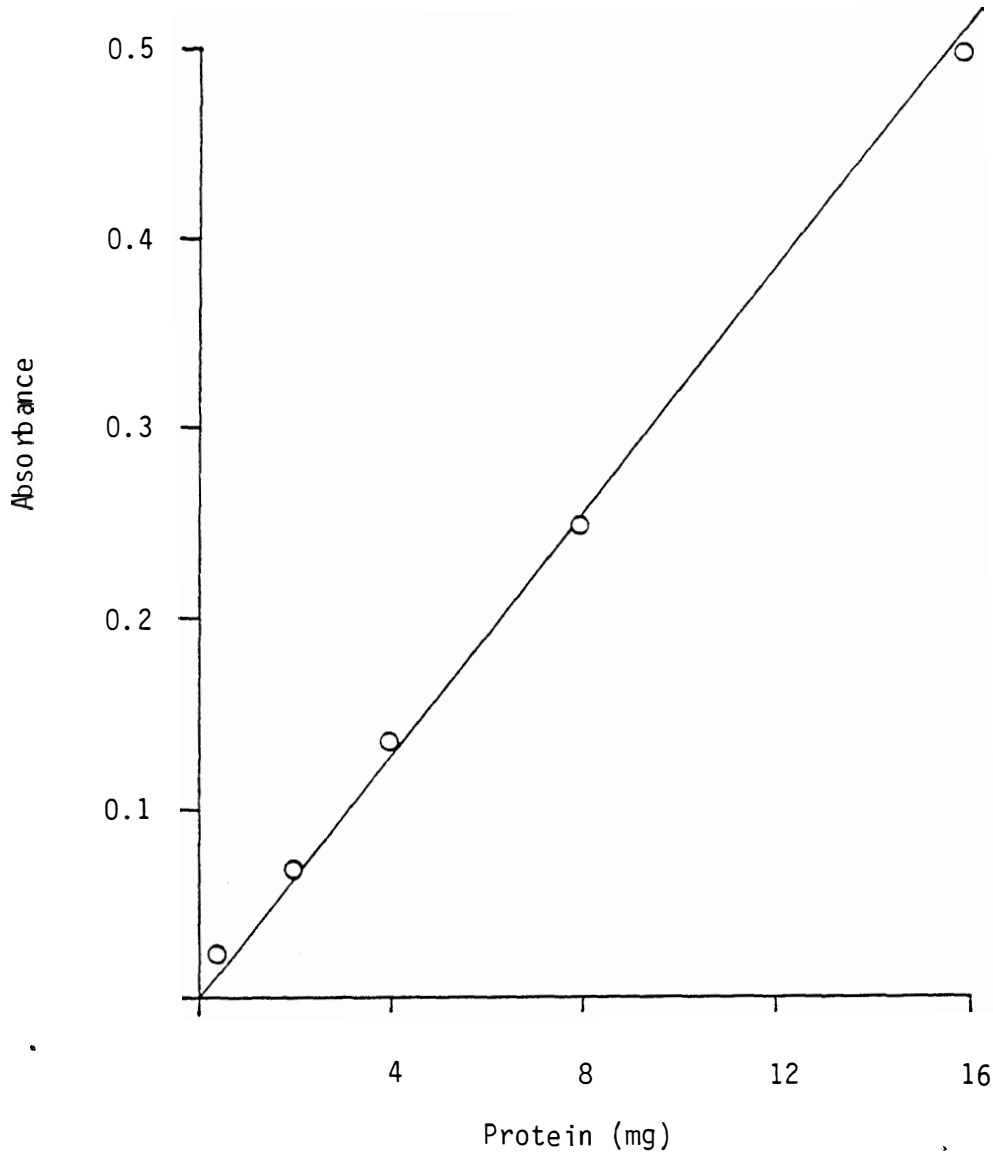


Figure 3. Standard curve for 100,000 x g SN protein obtained with BSA at 540 nm.

3. 0.3 M potassium phosphate (KPO_4) buffer: sufficient volume of 0.6 M KH_2PO_4 was added to 500 ml of 0.6 M K_2HPO_4 with continuous stirring at room temperature until the pH meter registered 7.8. The resultant volume was doubled with GDW and the solution was kept refrigerated.

4. Glycerol-phosphate buffer: glycerol (Certified A.C.S. grade, Fisher Scientific Co., Fairlawn, NJ) was mixed with an equal volume of 3 M KPO_4, pH 7.8 solution and kept refrigerated.

5. Sodium dithionite ($Na_2S_2O_4$).

Procedure. The microsomal suspension was diluted with glycerol-phosphate buffer to obtain a suspension with a concentration of 2 mg protein/ml. To each of two 4 ml quartz cuvettes, 2.5 ml of the diluted microsomal suspension and 15 mg of $Na_2S_2O_4$ was added. The suspension was mixed gently by inverting the cuvettes and after 2 minutes, the baseline at 450 nm was adjusted in a double beam spectrophotometer (Model 34, Beckman Instruments, Inc., Palo Alto, CA). Carbon monoxide was then bubbled through one of the cuvettes (sample cuvette) at approximately 40 bubbles per minute via a pasteur pipette tip for 60 seconds and the difference in absorbance at 450 nm and 490 nm against the reference cuvette at room temperature was recorded. The extinction coefficient of 91 $mM^{-1}cm^{-1}$ was used to calculate the concentrations:

$$\text{Cytochrome P-450 (nmoles/mg protein)} = \frac{A \times V \times 1{,}000}{E \times MP}$$

where:

A = difference in absorbance between 450 nm and 490 nm

V = volume of microsomal suspension in cuvette

E = extinction coefficient (91 $mM^{-1}cm^{-1}$)

MP = mg of microsomal protein present in the cuvette

NADPH-Cytochrome C Reductase Activity

The in vitro NADPH-cytochrome c reductase activity in the microsomes was determined according to the method of Williams and Kamin (80). The principle in this method involves the reduction of cytochrome c (which has an absorbance spectrum at 550 nm) by the enzyme in the presence of NADPH.

Reagents.

1. Glycerol-phosphate buffer: as described previously under cytochrome P-450 content determination.

2. Cytochrome c solution (4.4 g/ml): 44 mg of cytochrome c (Sigma Chemical Co., St. Louis, MO) was dissolved in 10 ml of glycerol-KPO_4 buffer, pH 7.8. The solution was kept at 0^oC.

3. 0.2 M sodium carbonate: 1.06 g of anhydrous Na_2CO_3 was dissolved in 50 ml of GDW.

4. 0.2 M sodium bicarbonate: 0.840 g of $NAHCO_3$ was dissolved in 50 ml of GDW.

5. 0.1 M carbonate buffer, pH 10.4: 38.5 ml of the 0.2 M Na_2CO_3 was mixed with 11.5 ml of 0.2 M $NaHCO_3$ and the total volume was brought up to 100 ml with GDW.

6. Nicotinamide adenine dinucleotide phosphate (NADPH) solution (8.6 mg/0.5 ml): 8.6 mg of NADPH was dissolved in 0.5 ml of

the 0.1 M carbonate buffer, pH 10.4 and the solution was kept on ice. This solution was prepared fresh daily.

Procedure. The diluted microsomal suspension (2 mg protein per ml) as described under the cytochrome P-450 assay, was used in this assay. To each of two 4 ml quartz cuvettes was added 1.6 ml of the 0.3 M KPO_4 buffer pH 7.8, 0.4 ml of the cytochrome c solution and 0.02 ml of the diluted microsomal suspension. The cuvettes containing the mixtures were gently mixed by inverting, and then zeroed on a double beam spectrophotometer. The reaction was started by the addition of 0.02 ml of the NADPH solution to the sample cuvette at room temperature. The sharp increase in absorbance at exactly 550 nm was recorded over time on the plotter and the change in absorbance per minute was determined by the slope of the line. The extinction coefficient of reduced cytochrome c of 21 $mM^{-1}cm^{-1}$ was used in the calculations:

$$\text{Cytochrome c reductase activity (nmole cytochrome c reduced/min/mg protein)} = \frac{\Delta A \times V \times 1{,}000}{E \times MP}$$

where:

ΔA = change in absorbance per minute at 550 nm

V = volume of mixture in the cuvette

E = extinction coefficient (21 $mM^{-1}cm^{-1}$)

MP = mg of microsomal protein present in the cuvette

Microsomal Aniline Hydroxylase Activity

The in vitro microsomal aniline hydroxylase activity was determined according to the procedure of Imai and Sato (81).

The principle in this procedure involves the enzymatic hydroxylation of aniline by the mixed function oxidase system in the microsomes to yield 4-OH-aniline (p-aminophenol). The protein fraction of the incubation mixture was then precipitated by trichloroacetic acid (TCA). The PAP was converted to indophenol and measured spectrophotometrically.

$$\text{aniline} \xrightarrow{\text{MFO}} \text{p-aminophenol} \longrightarrow \text{indophenol}$$

Reagents.

1. 0.2 M KPO_4., pH 7.4: prepared as described under 6PGD assay.

2. 0.2 M G6P: 0.2821 g of G6P-Na was dissolved in 5.0 ml of GDW.

3. 0.1 M NADP: prepared as described under 6PGD assay.

4. 0.5 M nicotinamide: 1.221 g of nicotinamide (niacinamide) (Sigma Chemical Co., St. Louis, MO) was dissolved in GDW and brought to a total volume of 20 ml.

5. 0.5 M $MgCl_2$: 5.0825 g of $MgCl_2.6H_2O$ was dissolved in GDW and brought up to a volume of 50 ml.

6. Stock G6PD (220 units/ml): 1 mg of solid G6PD (220 units) (Sigma Chemical Co., St. Louis, MO) was dissolved in 1 ml of 0.01 M KPO_4, pH 7.4.

7. G6PD (22 units/ml): 0.25 ml of the stock G6PD was diluted to 2.5 ml with 0.01 M KPO_4, pH 7.4.

8. 0.01 M KPO_4 buffer, pH 7.4: 5 ml of the 0.2 M KPO_4, pH 7.4 was diluted to 100 ml with GDW.

9. 0.1 M aniline: 0.92 ml of aniline hydrochloride (Sigma Chemical Co., St. Louis, MO) was diluted to 100 ml with GDW.

10. 10% (w/v) trichloroacetic acid (Cl_3CCO_2H): 100 g of Cl_3CCO_2H (TCA) (Fisher Scientific Co., Fair Lawn, NJ) was dissolved in GDW and the total volume brought up to one liter.

11. 10% Na_2CO_3: 10 g of Na_2CO_3 was dissolved in GDW and the total volume brought up to 100 ml.

12. 0.2 N NaOH-2% phenol: 2 g of phenol was dissolved in 10 ml of 2 N NaOH and the total volume brought up to 100 ml with GDW.

13. NADPH generating system: a NADPH-generating system was prepared to deliver in each 0.63 ml aliquot, 0.15 ml of G6P (#2), 0.3 ml of NADP (#3), 0.05 ml of nicotinamide (#4), 0.03 ml of $MgCl_2$ (#5) and 0.1 ml of G6PD (#7).

14. 0.1 M p-aminophenol (PAP): 0.1093 g of p-aminophenol ($NH_2C_6H_4OH$) (Fisher Scientific Co., Fair Lawn, NJ) was dissolved in 100 ml of 95% ethanol.

Procedure. The reaction mixture in a 25 ml Erlynmeyer flask contained 1.5 ml of the 0.2 M KPO_4 buffer, 0.63 ml of the NADPH generating system, 0.3 ml of the microsomal suspension (approximately 3 mg protein) and 0.42 ml of GDW. This was preincubated at 37^{o}C in a shaking water bath (100 shakes per minute). The reaction was initiated by the addition of 0.15 ml of 0.1 M aniline. At the end of

the 15-minute incubation period, 1 ml of the 10% TCA was added to terminate the reaction.

The resultant mixture was chilled on ice and then centrifuged at 2,000 x g for ten minutes at 4°C. 1 ml of the TCA supernatant was added to 0.5 ml of 10% Na_2CO_3 followed by 1 ml of 0.2 N NaOH containing 2% phenol. This mixture was allowed to stand for 30 minutes at room temperature before the absorbance at 630 nm was determined. A standard curve was determined using five concentrations (1, 5, 10, 20 and 30 µl) of 0.01 M PAP and bovine serum albumin (3 mg protein per volume) in place of the microsomal suspension. A standard curve is shown in Figure 4. The calculations for the enzyme activity are as follows:

Aniline hydroxylase activity (nmoles of PAP formed/min/mg protein) = AU/AS x CS x DF/T

where:

AU = absorbance of unknown

AS = absorbance of standard

CS = concentration of standard

DF = dilution factor

T = incubation time in minutes

Microsomal p-Chloro-N-Methylaniline (PCMA) Demethylase Activity

The PCMA-N-demethylase activity in the microsomes was determined according to the procedure of Kupfer and Bruggeman (82). The principle of this assay involves the enzymatic N-demethylation of PCMA by the mixed function oxidase system in the microsomes to yield

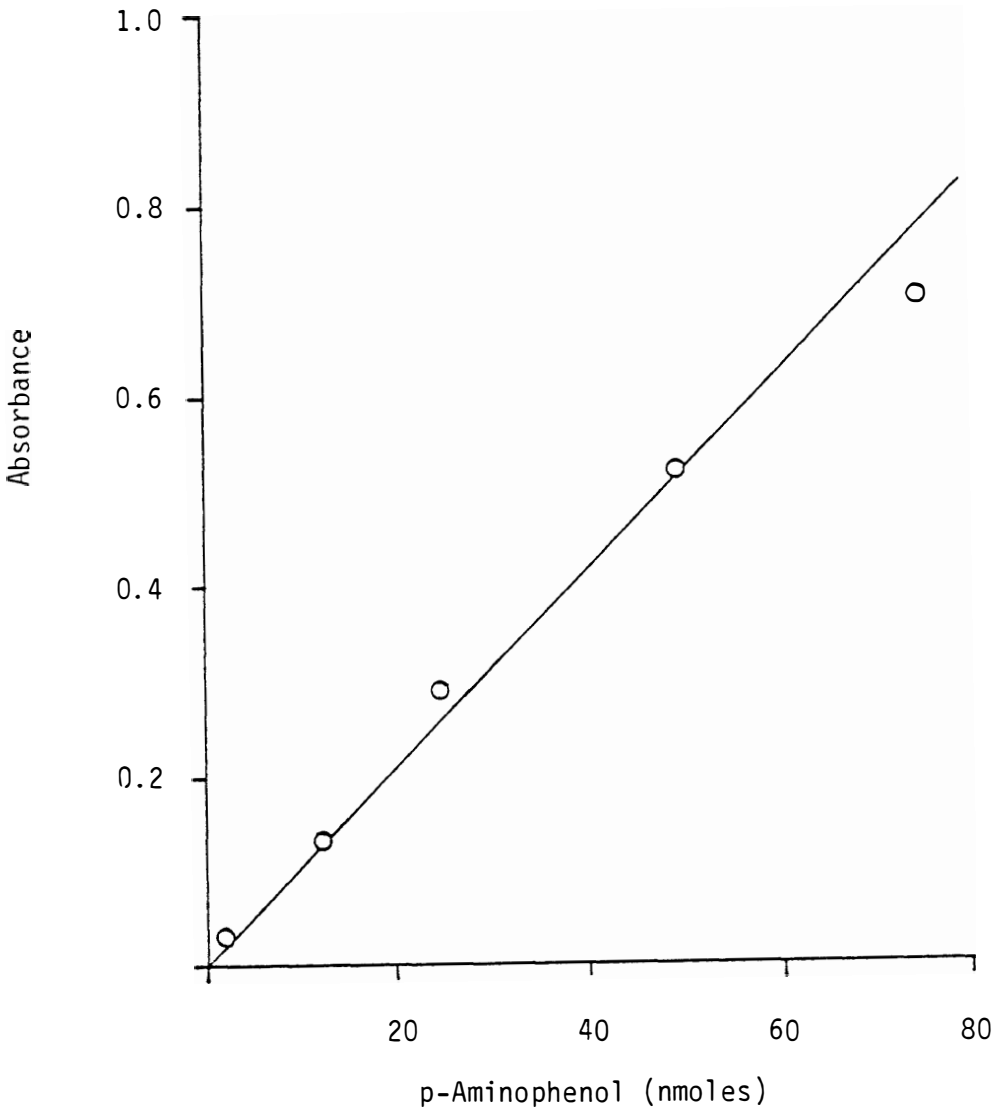
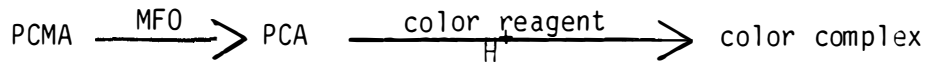


Figure 4. Standard curve for aniline hydroxylase assay obtained with p-aminophenol at 630 nm.

p-chloroaniline (PCA). The product is then reacted with a color reagent, p-dimethylamine-benzaldehyde, to form a color complex that absorbs light at 450 nm.



Reagents.

1. 0.1 M tris-HCl buffer, pH 7.4: 3.0275 g of Tris(hydroxymethyl)aminomethane (Trizma Base) (Sigma Chemical Co., St. Louis, MO) was dissolved in approximately 200 ml of GDW and the pH adjusted to 7.4 with 6N HCl. Then the total volume was brought up to 250 ml with GDW.

2. 0.2 M G6P: prepared as described under aniline hydroxylase assay.

3. 0.15 M KCl: 0.5592 g of KCl was dissolved in 50 ml of GDW.

4. 0.5 M $MgCl_2$: prepared as described under aniline hydroxylase assay.

5. 0.01 M NADP: prepared as described under 6PGD assay.

6. G6PD (22 units/ml): prepared as described under aniline hydroxylase assay.

7. 0.125 M PCMA: 0.038 ml of p-chloro-N-methylamine (Sigma Chemical Co., St. Louis, MO) was added to approximately 5 ml of GDW containing 0.05 ml of 6 N HCl and the total volume brought up to 10 ml. This solution was stored in the dark in the refrigerator since it is light-sensitive.

8. Color reagent: 6 g of p-dimethylaminobenzaldehyde (Eastman Kodak Chemical Co., Rochester, NY) was added to approximately 50 ml

of GDW and 8.32 ml of concentrated sulfuric acid (H_2SO_4). The total volume was then brought up to 100 ml with GDW.

9. Incubation mixture: an incubation mixture was prepared to deliver in a 4.51 ml aliquot, 2.5 ml of 0.1 M tris-HCl buffer, 0.06 ml of 0.2 M G6P, 0.4 ml of 0.15 M KCl and 1.55 ml of GDW.

Procedure. In a 25 ml Erlynmeyer flask the following was added: 4.51 ml of incubation mixture, 2 mg of microsomal protein, 0.05 ml of 0.5 M $MgCl_2$, 0.03 ml of 0.01 M NADP, 0.03 ml of 22 units/ml G6PD and GDW to bring the total volume to 5 ml while allowing room for 0.03 ml of 0.03 M PCMA. The mixture was preincubated at 37^{o}C for 5 minutes in a shaking (100 shakes per minute) water bath. The reaction was initiated by adding the substrate (0.03 ml of 0.03 M PCMA). At the end of the 20-minute incubation period, the reaction was terminated by the addition of 2 ml of the color reagent. The resultant mixture was centrifuged at 12,000 x g for 10 minutes and the absorbance of the supernatant determined at 445 nm against a blank prepared by adding the substrate after the reaction was terminated. To prevent any possible color stratification due to the high centrifugation, the supernatant was pipetted off into another tube and mixed gently by inverting before the absorbance determination. The extinction coefficient of 15.209 $mM^{-1}cm^{-1}$ was used in the calculation.

PCMA-N-demethylase activity (nmoles/min/mg protein)

$$= \frac{A \times DF \times 1{,}000}{E \times MP \times T}$$

where:

A = absorbance of the sample

E = extinction coefficient (15.209 $mM^{-1}cm^{-1}$)

MP = mg of microsomal protein

DF = dilution factor

T = incubation time in minutes

Resuspension of Microsomes

In our investigations, we observed that the microsomes suspended in the glycerol-0.15 M KPO_4 buffer did not provide adequate UDP-glucuronyl transferase activities. However, when the same microsomes were resuspended in 0.154 M KCl, the UDP-glucuronyl transferase activity was directly proportional to the protein content of microsomes in the assay mixture (unpublished observations).

Therefore, all the microsomes suspended in the glycerol-0.15 M KPO_4 buffer were resuspended in 0.154 M KCl as follows. An aliquot of 0.8 ml of the microsomes suspended in glycerol-0.15 M KPO_4 buffer was taken in an ultracentrifuge tube and 0.154 M KCl was added to bring the total volume to 10 ml. This suspension was gently mixed by inverting the tubes and then centrifuged at 100,000 x g for 1 hr. at 4^oC. The supernatant was decanted and the top of the pellet was rinsed with 1 ml of 0.154 M KCl without disturbing the pellet. Then the microsomal pellet was resuspended in 1 ml of 0.154 M KCl.

Protein Determination of 0.154 M KCl Suspended Microsomes

The protein determination of 0.154 M KCl resuspended microsomes was done according to the procedure of Lowry et al. (79).

All steps done were similar to the procedure described earlier for the microsomal suspension except that the 0.154 M KCl suspended microsomes were assayed instead of the glycerol-0.15 M KPO_4 buffer suspended microsomes. During the resuspension of the microsomes, some protein content may be lost. Thus the protein content of the new suspension (in 0.154 M KCl) was determined in order to facilitate calculations based on per mg protein or per gram liver.

Microsomal p-Nitrophenol UDP-Glucuronyl Transferase Activity

The microsomal UDP-glucuronyl transferase activity was determined according to the procedure of Graham and Wood (83).

The principle of this procedure involves UDP-glucuronyl transferase (UDP-GT) catalyzed transfer of a glucuronyl residue from uridinediphosphoglucuronate (UDPGA) to an aglycone (p-nitrophenol) to form the corresponding glucuronide and uridine phosphate (UDP). p-Nitrophenol is used as the phenolic substrate and the enzyme activity is measured as the disappearance of p-nitrophenol which has an absorbance peak at 400 nm.

$$\text{UDPGA} + \text{aglycone} \xrightarrow{\text{UDP-GT}} \text{glucuronide} + \text{UDP}$$

Reagents.

1. 8.33×10^{-2} M K_2HPO_4: 1.4512 g of K_2HPO_4 was dissolved in GDW and the total volume brought up to 100 ml.

2. 8.33×10^{-2} M KH_2PO_4: 1.1341 g of KH_2PO_4 was dissolved in GDW and the total volume was brought up to 100 ml.

3. 8.33×10^{-2} M KPO_4, pH 7.1: the 8.33×10^{-2} M KH_2PO_4 was added to 100 ml of 8.33×10^{-2} M K_2HPO_4 to get a pH of 7.1.

4. Reaction mixture B: 1.854 mg of p-nitrophenol was dissolved in 20 ml of 8.33×10^{-2} M KPO_4, pH 7.1.

5. Reaction mixture A: 0.0232 g of UDPGA ammonium salt (Sigma Chemical Co., St. Louis, MO) was dissolved in 6 ml of reaction mixture B.

6. 2% TCA: 10 g of TCA was dissolved in 500 ml of GDW.

7. 10 N KOH: 285 g of KOH was dissolved in GDW and the total volume brought up to 500 ml.

Procedure. A 0.2 ml aliquot of the 0.154 M KCl suspended microsomes was added to each of two test tubes containing 0.3 ml of either reaction mixture A or reaction mixture B that had been preincubated at 37°C for three minutes. The reaction was allowed to proceed at 37°C with agitation for 30 minutes before being terminated by the addition of 4 ml of 2% TCA. This suspension was then centrifuged at 760 x g for ten minutes at 4°C. A 1 ml aliquot of the supernatant thus obtained was added to another tube containing 0.06 ml of 10 N KOH. The absorbance of the sample was read at 400 nm against a blank containing reaction mixture B and 0.2 ml of GDW. A standard curve using six volumes (15, 75, 113, 150, 225 and 300 μl) of 6.67×10^{-4} M p-nitrophenol instead of the sample in reaction mixture B is shown in Figure 5. The calculations for the p-nitrophenol UDP-glucuronyl transferase activity are as follows:

$$\text{UDP-glucuronyl transferase activity (nmoles of p-nitrophenol conjugated/min/mg protein} = \frac{AB - AA}{AS} \times CS \times DF$$

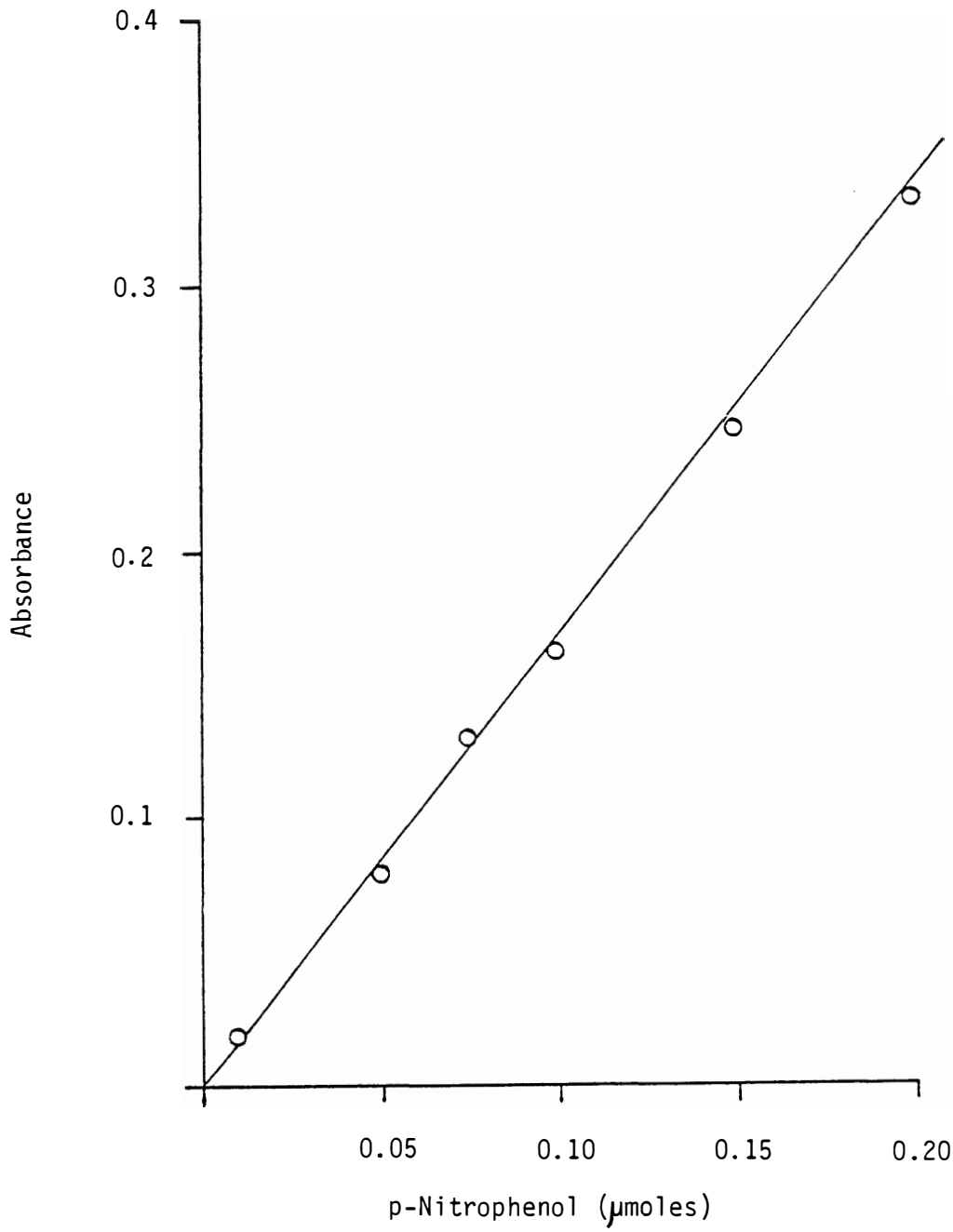


Figure 5. Standard curve for UDP-glucuronyl transferase assay obtained with p-nitrophenol at 400 nm.

where:

AB = absorbance of the sample in reaction mixture B

AA = absorbance of the sample in reaction mixture A

AS = absorbance of the standard

CS = concentration of the standard

DF = dilution factor

6-Phosphogluconate Dehydrogenase Determination in the 100,000 x g SN

The determination of 6-phosphogluconate dehydrogenase (6PGD) activity is based on formation of NADPH (measured by the increase in absorbance at 340 nm) when 6-phospho-D-gluconate (6PG) is oxidized to ribulose-5-phosphate as follows:

$$\text{6-phospho-D-gluconate} + NADP^{+} \xrightarrow{\text{6PGD}} \text{ribulose-5-phosphate} + NADPH + H^{+} + CO_2$$

The procedure of Glock and McLean (85) was employed with some modifications. It was previously determined in our laboratory that in an unpurified system such as the 100,000 x g SN, the addition of NADP alone to the reaction mixture in the sample cuvette containing the glycylglycine buffer, $MgCl_2$ and 100,000 x g SN without the 6PG substrate resulted in the endogenous enzyme activity as measured by increasing absorbance at 340 nm. This would result in a higher reading and thus an erroneous indication of the enzyme content. Thus, in our procedure, NADP was added to both cuvettes (to account for the other endogenous substrates) and the reaction was initiated by the addition of 6PG instead of the NADP.

Reagents.

1. 0.25 M glycylglycine buffer, pH 7.6: 1.053 g of glycylglycine hydrochloride (Sigma Chemical Co., St. Louis, MO) was dissolved in approximately 15 ml of GDW. The pH was then adjusted to 7.6 with 2 N NaOH. The total volume was brought up to 25 ml and the solution kept refrigerated.

2. 0.4 M K_2HPO_4: 13.93 g of K_2HPO_4 was dissolved in GDW and the total volume brought up to 200 ml.

3. 0.4 M KH_2PO_4: 10.89 g of KH_2PO_4 was dissolved in GDW and the total volume brought up to 200 ml.

4. 0.2 M KPO_4 buffer, pH 7.4: 0.4 M KH_2PO_4 was added to 200 ml of the 0.4 M K_2HPO_4 until a pH of 7.4 was obtained. The resultant volume was then doubled with distilled water.

5. 0.01 M nicotinamide adenine dinucleotide phosphate (NADP): 37.2 g of NADP (Sigma Chemical Co., St. Louis, MO) was dissolved in 5 ml of 0.2 M KPO_4 buffer pH 7.4 and stored at 0^oC.

6. 2.7 mM NADP: 2.7 ml of the 0.01 M NADP was diluted to 10 ml with 0.2 M KPO_4, pH 7.4 and stored at 0^oC.

7. 0.5 M magnesium chloride ($MgCl_2$): 10.16 g of $MgCl_2.6H_2O$ was dissolved in GDW and the total volume brought up to 100 ml.

8. 0.05 M 6-phosphogluconic acid trisodium salt: 85.5 mg of 6PG-Na_3 (Sigma Chemical Co., St. Louis, MO) was dissolved in 5 ml of GDW and stored in the refrigerator.

Procedure. To each of two 4 ml quartz cuvettes 0.5 ml of the 0.25 M glycylglycine buffer, pH 7.6, 0.1 ml of the 2.7 mM NADP, 0.1 ml of the 0.5 M $MgCl_2$ and 0.05 ml of the 100,000 x g SN was added. In

the reference cuvette, 2.25 ml of GDW was added to bring the total volume to 3 ml and in the sample cuvette, 2.15 ml of GDW was added to allow room for the addition of the substrate. The mixtures were mixed gently by inverting the cuvettes and then zeroed in a double beam spectrophotometer. The reaction was started by the addition of 0.1 ml of 0.05 M 6PG-Na_3 and the change in absorbance over time at 340 nm at room temperature was recorded on the chart plotter. The change in absorbance per minute at 340 nm was determined from the plot and the extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ was used in the calculations:

$$\text{6PGD activity (nmoles NADPH produced/min/mg protein)} = \frac{\Delta A \times V \times 1{,}000}{E \times MP}$$

where:

ΔA = change in absorbance per minute at 340 nm

V = volume of reaction mixture in the cuvette

E = extinction coefficient (6.22 $mM^{-1}cm^{-1}$)

MP = mg of 100,000 x g SN protein in the cuvette

Glucose-6-Phosphate Dehydrogenase Determination in the 100,000 x g SN

The glucose-6-phosphate dehydrogenase (G6PD) activity was determined according to the procedure of Glock and McLean (85). In this procedure, the combined activity of G6PD and 6PGD in the presence of saturated concentrations of glucose-6-phosphate (G6P) and 6PG were determined together and then the activity of 6PGD determined previously was subtracted from the combined activities.

The reaction included the formation of NADPH from the oxidation of G6P as well as those from the oxidation of 6PG. The oxidation of G6P is as follows:

$$\text{Glucose-6-phosphate} + \text{NADP}^{+} \xrightarrow{\text{G6PD}} \text{gluconolactone-6-phosphate} + \text{NADPH} + \text{H}^{+}$$

Reagents.

1. 0.2 M G6P: 0.2821 g of G6P-Na (Sigma Chemical Co., St. Louis, MO) was dissolved in 5 ml of GDW.

2. 0.05 M G6P: 2.5 ml of the 0.2 M G6P was diluted to 10 ml with GDW.

3. Other reagents were the same as those used for the 6PGD assay.

Procedure. The assay procedure was similar to that described for the assay of 6PGD activity except for the addition of 0.1 ml of 0.5 M G6P and 0.1 ml of 0.5 M 6PG as substrates instead of just 6PG. The amount of GDW added was adjusted accordingly to obtain a total volume of 3 ml in the cuvettes. The change in absorbance per minute at 340 nm was determined and the combined activities of G6PD and 6PGD were calculated as follows:

$$\text{G6PD + 6PGD activity (nmoles NADPH produced/min/mg protein)} = \frac{\Delta A \times V \times 1{,}000}{E \times MP}$$

where:

ΔA = change in absorbance per minute at 340 nm

V = volume of reaction mixture in the cuvette

E = extinction coefficient (6.22 $mM^{-1}cm^{-1}$)

MP = mg of 100,000 x g SN protein in the cuvette

The activity of G6PD was calculated by subtracting the value for the 6PGD activity determined previously from the combined G6PD + 6PGD activities.

Malic Enzymes Determination in the 100,000 x g SN

The activity of malic enzymes (ME) was determined according to the procedure of Ochoa (86).

The reaction involves the conversion of L-malate to pyruvate resulting in the formation of NADPH which is measured by the increase in absorbance at 340 nm:

$$\text{L-malate} + NADP^+ \xrightarrow{ME} \text{pyruvate} + CO_2 + NADPH + H^+$$

Our procedure again was modified slightly to account for the reaction occuring after the addition of NADP to the mixture. NADP was added to both cuvettes and the reaction was initiated by the addition of L-malate.

Reagents.

1. 0.03 M L-malate-Na: 0.2012 g of L-malate-Na (Sigma Chemical Co., St. Louis, MO) was dissolved in approximately 30 ml GDW and the pH was adjusted to 7.0 with 2 N NaOH. The total volume was brought up to 50 ml with GDW.

2. 0.01 M NADP in glycylglycine buffer, pH 7.6: 37.2 mg of NADP was dissolved in 5 ml of 0.25 M glycylglycine buffer, pH 7.6 and stored at 0^oC.

3. 2.7 mM NADP: 2.7 ml of the 0.01 M NADP was diluted to 10 ml with the glycylglycine buffer, pH 7.6.

4. 0.25 M glycylglycine buffer, pH 7.6: prepared as described previously under 6PGD assay.

5. 0.05 M manganese chloride ($MnCl_2$): 0.1979 g $MnCl_2$ was dissolved in 20 ml GDW.

Procedure. To each of the two 4 ml quartz cuvettes 0.3 ml of the 0.25 M glycylglycine buffer, 0.06 ml of the 0.05 M $MnCl_2$, 0.05 ml of NADP and 0.05 ml of the 100,000 x SN was added to each cuvette to bring the total volume to 3 ml except that in the sample cuvette, room was allowed for the addition of 0.05 M L-malate. The reaction was initiated by the addition of 0.05 ml of the 0.05 M L-malate and the change in absorbance at 340 nm per minute was determined. The calculations for the ME activity were as follows:

ME activity (nmoles NADPH produced/min/mg protein)

$$= \frac{\Delta A \times 3 \times 1{,}000}{E \times MP}$$

where:

ΔA = change in absorbance at 340 nm per minute

E = extinction coefficient (6.22 $mM^{-1}cm^{-1}$)

MP = mg 100,000 x g SN protein in cuvette

2. EXPERIMENT II

Thirty male weanling Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing approximately 60 g were divided into two groups of ten animals each. Animals were housed in facilities as

described for Experiment 1 and fed the same semi-synthetic diet. The animals in the first group (ad lib group) were fed the semi-synthetic diet ad libitum and the animals in the second group (45% restricted group) were given the same diet at an amount equal to 55% of that consumed by the ad libitum group the previous day. Distilled water was made available to all animals ad libitum.

Cannulation of Right Jugular Vein

At the end of the four-week experimental period, the right external jugular vein of these animals were cannulated according to the procedure of Upton (87).

Surgical Equipment

1. Fine forceps.(Accurate Surgical & Scientific Instruments Corp., Westbury, NY.)
2. Fine scissors.(Accurate Surgical & Scientific Instruments Corp., Westbury, NY.)
3. Small scissors.
4. Suture needles.
5. Suture silk - 4-0.
6. Small needle holder.
7. Small forceps.
8. Trochar - 14 gauge.
9. Heparinized saline - 10 units/ml normal saline.
10. Silastic (602-135) tubing, i.d. 0.51 mm, o.d. 0.94 mm, - 150mm length with one end beveled at 45 , (Dow Corning International Ltd., Midland, MI).

11. Hypodermic needles - 24 gauge.

12. Disposable syringes - 1 cc.

13. Hematocrit sealing putty.

14. Hair clipper.

15. Neosporin antibacterial ointment, (Burroughs Wellcome Co., Research Triangle Park, NC).

16. Ether anaesthesia chamber.

17. Ethyl ether, (Fisher Scientific Co., Fair Lawn, NJ).

18. Resuscitator.

19. Alcohol swabs, (American Scientific Products, McGaw Park, IL).

Surgical Procedure

The rat was anaesthetized with ether and the hair was removed from about a 3 cm square area above the right jugular vein. The area was sterilized with an alcohol swab. During the rest of the surgical procedure, anaesthesia was maintained by applying a 20 ml beaker containing a small wad of surgical gauze saturated with ether over the rat's nose and mouth (care was taken to avoid suffocating the animal. An artificial resuscitation apparatus was constructed from a one way rubber bulb, rubber tubing and a plastic funnel. This was used for emergency resuscitation when needed).

The surgical procedure was carried out under aseptic conditions and all equipment were sterilized prior to the surgery. A small skin incision (approximately 1 cm) was made above the right jugular vein in an anterior direction starting from a point above the collar bone. By blunt dissection, approximately 75 mm of the right

jugular vein was exposed. A folded piece of suture thread was passed under the exposed vein and the loop cut to make two single strands of suture thread. The anterior end of the exposed vein was then ligated and the tie left uncut. The top surface of the exposed vein was picked up with a pair of fine forceps and a small obligue cut was made with a pair of fine scissors. The 150 mm silastic tubing was rinsed with heparinized saline before being inserted (beveled end first) into the vein and passed towards the heart (approximately 30-40 mm). The tubing was then secured by tying the posterior of the two strands of suture thread firmly around the vein just below the point of insertion and again with another suture thread around the vein just above of the collar bone. The exposed tubing was further secured to the depleted section of the vein by the ends of the anterior ligature. The tubing was then inserted via a trochar, under the skin at a point in line with but anterior to the exposed section of the vein and passed subcutaneously to exit at a point on the back of the neck between the scapulae. A syringe charged with heparinized saline was attached to the tubing. Possible occlusion of the cannula was monitored after each tie and after the subcantenous insertion of the tubing by moving the plunger. The surgical wound was then closed with four or five interrupted sutures and Neosporin antibacterial ointment was applied. After the injection of 0.2 ml heparinized saline, the cannula was cut to a length of approximately 3 cm and stoppered with a plug fashioned from a 24 gauge needle (the ends of the needle were plugged with hematocrit tube sealing putty). The

animal was then allowed to recover from the anaesthesia for 3-4 hr before being dosed with antipyrine.

Blood Antipyrine Clearance Determination

The blood clearance of antipyrine was determined according to the procedure of Knights et al. (88)

Reagents

1. Cyproheptadine internal standard, 0.4 mg/ml: 4 mg of cyproheptadine (Sigma Chemical Co., St. Louis, MO) was dissolved in 10 ml of GDW.

2. Methylene chloride: Fisher Certified ACS Grade (Fisher Chemical Co., Fair Lawn, NJ).

3. Antipyrine (20 mg/ml): 0.5 g of antipyrine (Sigma Chemical Co., St. Louis, MO) was dissolved in 25 ml of normal saline.

4. Antipyrine standard (2 mg/ml): 1 ml of the 20 mg/ml antipyrine was diluted to 10 ml with normal saline.

Procedure

Antipyrine (40mg/kg body weight) was injected into the rat via the cannula followed by a 0.2 ml flush of heparinized saline. Blood samples (0.2 ml) were collected at 15, 30, 60, 90, 120, 180 and 240 minutes post dosage as follows: Approximately 0.2 ml of blood was collected in a 1 cc syringe via the cannula and transferred immediately into an eppendorf tube. Exactly 0.1 ml aliquot of the blood was then immediately transferred into a heparinized eppendorf tube containing 10 μl of 0.4 mg/ml cyproheptadine, 100 μl of GDW and

50 µl of methylene chloride. (Fluid replacement was provided with 0.2 ml of normal saline after each sampling). The mixture was vortexed for 1 minute and centrifuged in an eppendorf centrifuge for 2 minutes. The organic phase (1 µl) was injected into a gas liquid chromatograph (HP 5840A, Hewlett-Packard Instrument Co., Mountain View, CA) fitted with a 2 m x 2mm ID glass column packed with 3% OV-25 on Gas Chrom Q - 80/100 mesh (Supelco Inc., Bellefonte, PA). The output for the nitrogen-phosphorus detector was monitored on the 5840A integrator. The temperatures for the injection port, column and detector were 250^{o} , 250^{o} , and 300^{o}C respectively. The flow rates for the helium carrier gas, detector hydrogen and air were 20, 3 and 50 ml/minute respectively.

A standard curve was prepared by adding the antipyrine standard to yield concentrations of 0, 0.5, 1, 2, 3 and 5 µg/100 µl of whole blood (Figure 6). The antipyrine concentration was determined by comparing the area ratio of antipyrine to cyproheptadine in the sample with that of the antipyrine to cyproheptadine in the standard. The calculations were as follows:

$$\text{Antipyrine } (\mu g/100\ \mu l \text{ of blood}) = \frac{aAU \times aCS \times cAS}{aCU \times aAS}$$

where:

aAU = area ratio of antipyrine in sample.

aCU = area ratio of cyproheptadine in sample.

aAS = area ratio of antipyrine in standard.

aCS = area ratio of cyproheptadine in standard.

cAS = concentration of antipyrine standard.

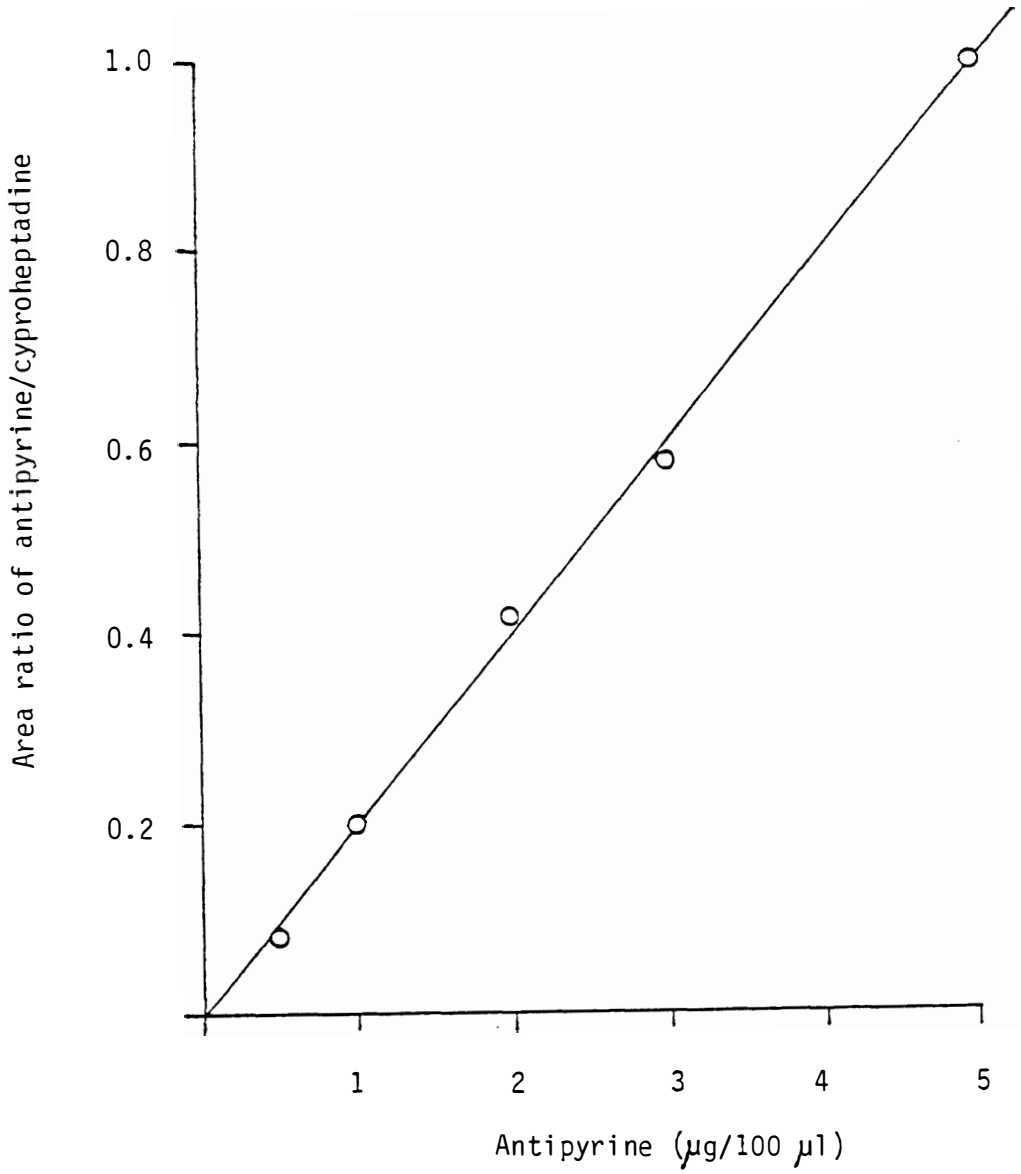


Figure 6. Standard curve for antipyrine.

Pharmacokinetic Analysis

Kinetic parameters of antipyrine were calculated according to the procedures described by Gibaldi (89).

The blood antipyrine data was plotted on semilogarithmic paper with the concentration of antipyrine in the blood on the y - axis (logarithmic scale) and the time of sampling on the x - axis (linear scale). The straight line obtained was extrapolated back to time zero to obtain C_o, the theoretical antipyrine concentration in the blood at time zero. The volume of distribution (V) was then calculated as follows:

$$V = \text{i.v. dose}/C_o$$

The elimination half-life ($T_{1/2}$) of antipyrine (i.e. the time required for the concentration to be reduced by 50%) was determined directly from the graph.

The elimination constant (k) was calculated as:

$$k = 0.693/T_{1/2}$$

The clearance rate (Cl) (amount of blood that is cleared of the drug in unit time) was calculated as:

$$Cl = k \times V$$

3. EXPERIMENT III

The animals in Experiment 3 were the same animals that were used for the antipyrine experiment. At approximately 36 - 42 days after the antipyrine experiment, the rats were gavaged with an oral LD_{50} (5.4 mg/kg body weight) dose of carbon tetrachloride (Fischer

Chemical Co., Fair Lawn, NJ). (The carbon tetrachloride was mixed with mineral oil in a 1:1 (v/v) solution). The LD_{50} dose of 5.4 mg/kg body weight was the lower range of the LD_{50} dose for rats fed ad libitum determined by Mclean and Mclean (90).

The mortality was monitored for one week before the surviving animals were killed by sodium brevital administration.

4. STATISTICAL METHODS

The effects of different levels and duration of feed restriction on the various drug metabolism parameters were analyzed utilizing the procedures available in GLM of SAS 1982 (91). For each drug metabolism parameter, differences among the treatment groups were evaluated using the analysis of variance. Significant parameters were tested for specific effects of level and duration of feed restriction to determine if the relationship was linear, quadratic or cubic. For those parameters where no significant effects of level and duration of feed restriction were observed among the treatment groups, the t-test was utilized to compare the mean of all treatment groups with the mean of the ad libitum group. A probability level of 0.05 or less was considered statistically significant. The least squares option was utilized to derive the group means and standard errors.

In Experiments II and III, the Student's t-test was utilized to determine significant differences between the two treatment means at the level of $P \leq 0.05$.

CHAPTER IV

RESULTS

1. EXPERIMENT I

The effects of different levels and duration of feed restriction were examined in 15 groups of healthy rats. On any parameter examined, when no significant effect of feed restriction was found among the 15 treatment groups, the mean of all the treatment groups was then compared with that of the ad libitum group of rats. It must be pointed out that the values reported for the treatment groups represented changes in the parameters primarily during the period of restriction. In the description of the analysis, a linear relationship denotes a straight line relationship between two variables (for example, a linear relationship between feed consumption and body weight gain means that for each unit increase or decrease in feed consumption, one can expect a proportional unit increase or decrease in the body weight gain in a straight line manner). A quadratic relationship simply denotes that the relationship is curvilinear (with one curvature or bend in the plotted line) whereas a cubic relationship denotes a curve with two bends in the plotted line, usually one concave and one convex much like the plot of a sine wave.

When there is a significant effect of both the level and duration of feed restriction, a three dimensional graph is employed to depict the response of the dependent variable (parameter). This response surface allows one to see the effects of both independent

variables on the dependent variable at the same time. The equation for the response surface was derived using significant terms ($P \leq 0.05$) of the analysis as well as including those that would contribute to the significant terms (for example, if the quadratic duration by quadratic level interaction was significant, the other terms containing quadratic components of duration and level and all linear terms would also be included in the equation).

The total feed consumption data of these rats are presented in Table 3. The decrease in feed consumption with increasing levels and duration of feed restriction was significant and follows an expected pattern (Table 4). Upon statistical analysis of the data, the feed consumption of the restricted animals decreased in a significant linear manner with increasing levels and a quadratic manner with the increasing duration of feed restriction. The significant quadratic duration by linear level interaction observed introduced a slight curvature on the duration side of the response surface (Figure 7). Thus, as the level of feed restriction increased, the total feed consumed decreased linearly and as the duration of feed restriction increased, the total feed consumed decreased curvilinearly. The equation for the response surface is as follows:

$$\text{Feed consumption (g)} = 591.94 - 46.69\ D - 2.26\ L + 0.86\ DL + 7.75\ D^2 - 0.30\ D^2L$$

where: D = duration of feed restriction

TABLE 3

FEED CONSUMPTION[1] BY LEVEL AND DURATION OF FEED RESTRICTION

Level of restriction	Duration of restriction (weeks)					
	0	1	2	3	4	5
	(g)					
0%	543.7 ± 5.5[2]					
15%		522.3 ± 10.3	500.3 ± 10.3	479.3 ± 10.4	481.3 ± 10.4	468.3 ± 10.3
30%		509.0 ± 9.4[3]	494.7 ± 10.3	448.0 ± 11.7[4]	419.6 ± 10.3	385.7 ± 10.4
45%		467.5 ± 10.3	454.3 ± 10.4	397.3 ± 10.4	371.8 ± 10.3	301.4 ± 10.4

[1] Unless otherwise stated, each value is the LSMEAN ± SEM of 5 animals.

[2] n = 17 animals.

[3] n = 6 animals.

[4] n = 4 animals.

TABLE 4

F VALUE FROM THE ANALYSIS OF VARIANCE FOR THE EFFECTS OF DIFFERENT LEVELS AND DURATION OF FEED RESTRICTION ON FEED CONSUMPTION, FINAL BODY WEIGHT, WEIGHT GAIN AND LIVER WEIGHT

Source of variation	df	F Value			
		feed consumption	final body weight	weight gain	liver weight
BLOCKS	9	21.98[1]	6.98[1]	5.50[1]	10.58[1]
DURATION					
linear	1	317.21[1]	28.67[1]	29.44[1]	21.72[1]
quadratic	1	1.02	0.22	0.67	1.03
cubic	1	0.01	0.20	0.16	0.01
LEVEL					
linear	1	193.49[1]	40.46[1]	44.68[1]	151.90[1]
quadratic	1	2.74	6.85[1]	4.30[1]	1.62
DURATION X LEVEL INTERACTIONS					
Duration x level	1	56.68[1]	11.47[1]	12.65[1]	5.16[1]
Duration^2 x level	1	7.95[1]	1.17	0.68	2.75
Duration x level^2	1	2.03	3.98	2.69	6.66[1]
Duration^2 x level^2	1	0.15	4.75[1]	4.00[1]	0.10
Duration^3 x level	1	0.36	2.08	0.90	3.93
Duration^3 x level^2	1	0.92	0.04	0.07	0.54

[1] $P \leq 0.05$.

[2] Quadratic function.

[3] Cubic function.

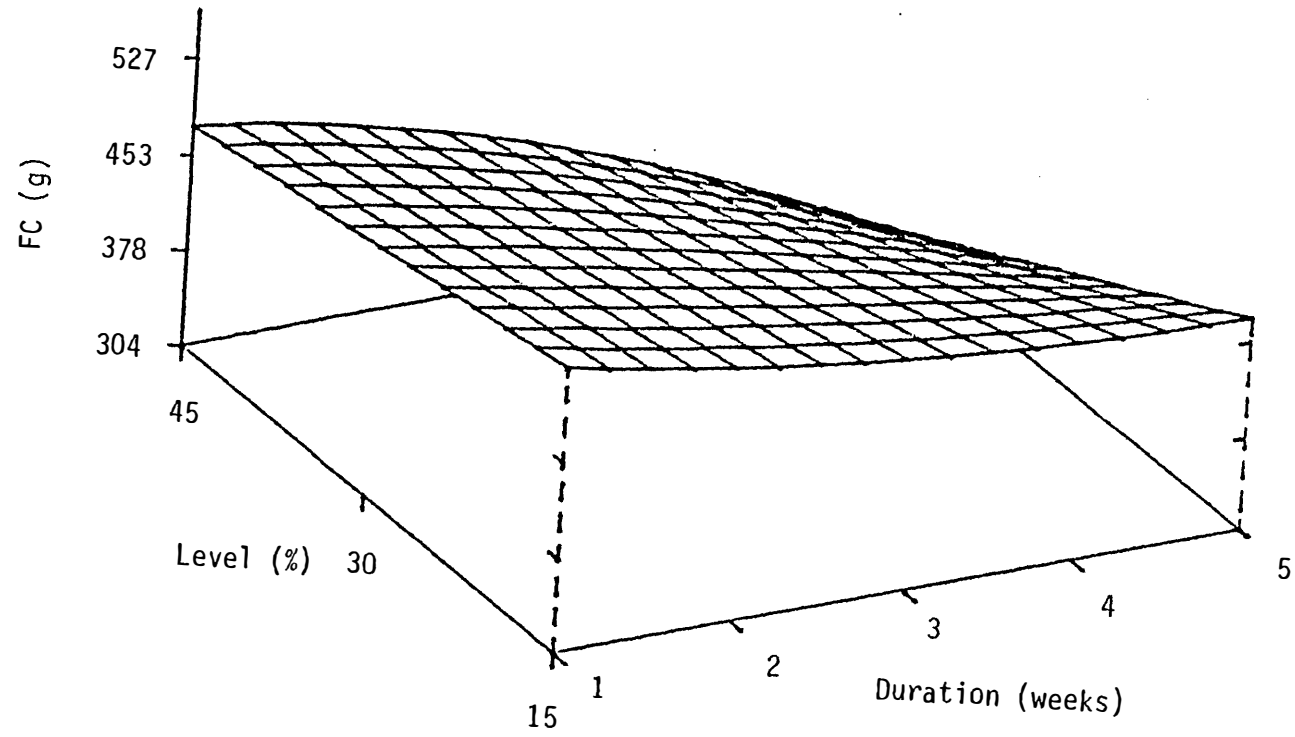


Figure 7. Feed consumption (FC) of animals at the different levels (L) and duration (D) of feed restriction. $FC = 591.94 - 46.69\ D - 2.26\ L + 0.86\ DL + 7.75\ D^2 - 0.30\ D^2L$ ($P \leq 0.05$).

and L = level of feed restriction (For the rest of this paper, duration and level in an equation will be denoted as D and L, respectively).

The data on final body weight, weight gain, feed consumption and liver weight are shown in Tables 5 through 7, respectively. Among the treatment groups, increasing levels and duration of feed restriction resulted in expected decreases in these four parameters. The decreases appeared to follow similar trends. Upon statistical analysis of the data, final body weight decreased in a significant curvilinear manner with increasing levels and duration of feed restriction (Table 4). In the analysis, highly significant linear components of duration and level effects on final body weight were observed as well as a significant quadratic component of the level effect. Significant interactions included linear duration by linear level and quadratic duration by quadratic level effects. The response surface is depicted in Figure 8 and the decrease in final body weight was greatest at the 45% level of feed restriction for five weeks. (Final body weight (g) = $164.33 + 73.15\ D + 6.97\ L - 6.46\ DL - 14.78\ D^2 + 1.27\ D^2L - 0.13\ L^2 + 0.11\ DL^2 - 0.023\ D^2L^2$).

As expected, the amount of body weight gain decreased as the level and duration of feed restriction increased (Table 5). This decrease was significantly affected by the increase in level and duration of feed restriction (Table 4). The response surface was curvilinear in both directions of level and duration, with the least amount of body weight gain at 45% feed restriction for five weeks

TABLE 5

EFFECT OF DIFFERENT LEVELS AND DURATION OF FEED RESTRICTION ON FINAL BODY WEIGHT[1]

Level of restriction	Duration of restriction (weeks)					
	0	1	2	3	4	5
	(g)					
0%	262.1 ± 3.9[2]					
15%		242.7 ± 7.3	238.5 ± 7.3	228.6 ± 7.3	241.7 ± 7.3	221.2 ± 7.3
30%		238.7 ± 6.7[3]	229.9 ± 7.3	218.6 ± 8.3[4]	224.1 ± 7.3	227.5 ± 7.3
45%		222.0 ± 7.3	217.4 ± 7.3	217.6 ± 7.3	182.8 ± 7.3	169.3 ± 7.3

[1]Unless otherwise stated, each value is the LSMEAN ± SEM of 5 animals.

[2]n = 17 animals.

[3]n = 6 animals.

[4]n = 4 animals.

TABLE 6

EFFECT OF DIFFERENT LEVELS AND DURATION OF FEED RESTRICTION ON BODY WEIGHT GAIN[1]

Level of restriction	Duration of restriction (weeks)					
	0	1	2	3	4	5
	(g)					
0%	160.5 ± 3.8[2]					
15%		140.1 ± 7.2	140.1 ± 7.2	130.0 ± 7.2	141.0 ± 7.2	121.9 ± 7.2
30%		136.0 ± 6.5[3]	126.7 ± 7.2	121.2 ± 8.1[4]	118.8 ± 7.2	123.1 ± 7.2
45%		122.2 ± 7.2	112.8 ± 7.2	118.6 ± 7.2	81.1 ± 7.2	68.2 ± 7.2

[1] Unless otherwise stated, each value is the LSMEAN ± SEM of 5 animals.

[2] n = 17 animals.

[3] n = 6 animals.

[4] n = 4 animals.

TABLE 7

EFFECT OF DIFFERENT LEVELS AND DURATION OF FEED RESTRICTION ON LIVER WEIGHT[1]

Level of restriction	Duration of restriction (weeks)					
	0	1	2	3	4	5
	(g)					
0%	10.2 ± 0.15[2]					
15%		8.3 ± 0.28	7.7 ± 0.28	7.6 ± 0.28	7.7 ± 0.28	7.3 ± 0.28
30%		6.9 ± 0.25[3]	7.1 ± 0.28	6.9 ± 0.32[4]	6.7 ± 0.28[4]	6.8 ± 0.28
45%		6.2 ± 0.28	6.4 ± 0.28	6.0 ± 0.28	5.2 ± 0.28	4.8 ± 0.28

[1] Unless otherwise stated, each value is the LSMEAN ± SEM of 5 animals.

[2] n = 17 animals.

[3] n = 6 animals.

[4] n = 4 animals.

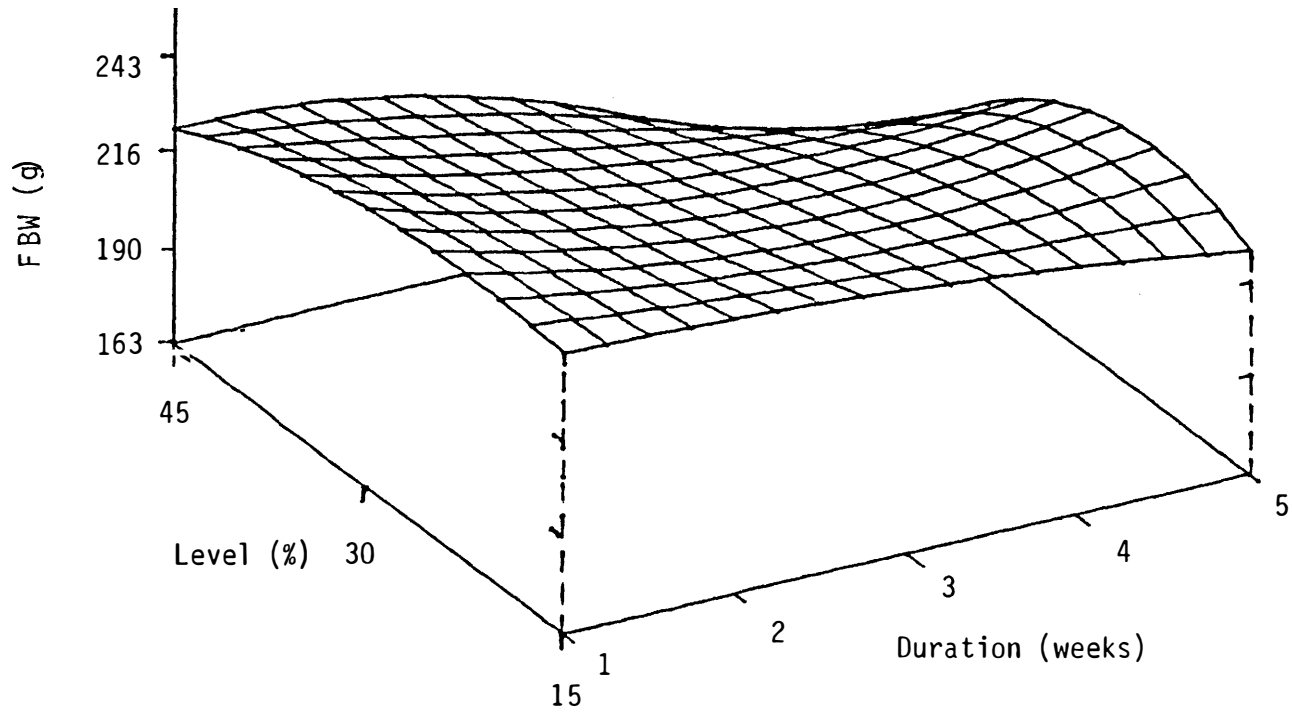


Figure 8. The effect of different levels (L) and duration (D) of feed restriction on final body weight (FBW). $FBW = 164.33 + 73.15\ D + 6.97\ L - 6.46\ DL - 14.78\ D^2 + 1.27\ D^2L - 0.13\ L^2 + 0.11\ DL^2 - 0.02\ D^2L^2$ ($P \leq 0.05$).

(Body weight gain (g) = 63.60 + 74.28 D + 6.52 L - 6.16 DL - 14.34 D^2 + 1.17 D^2L - 0.12 L^2 + 0.10 DL^2 - 0.021 D^2L^2) (Figure 9).

The decrease in liver weight also followed a pattern very similar to that of body weight gain (Table 7). The pattern of decrease with increasing levels and duration of feed restriction was significant (Table 4). Liver weight decreased curvilinearly as the level of feed restriction increased, and linearly as the duration of feed restriction increased (Figure 10). The lowest liver weight was observed at the highest levels and duration of feed restriction (Liver weight (g) = 10.59 - 0.76 D - 0.19 L + 0.054 DL + 0.0024 L^2 - 0.001 DL^2).

Increasing levels and duration of feed restriction did not alter the hepatic microsomal protein content in any specific pattern when expressed as per gram of wet liver tissue (Table 8). Thus, the mean hepatic microsomal protein content of the 15 treatment (feed restricted) groups were compared with that of the rats in the ad libitum group. The feed restricted rats had significantly higher mean hepatic microsomal protein content than the ad libitum controls (16.9 $\pm$ 0.25 vs 15.6 $\pm$ 0.40 mg/g liver, respectively) (Figure 11). When this protein is expressed as total hepatic content, a general decreasing pattern was observed with increasing levels and duration of feed restriction (Table 9). Upon statistical analysis, only the linear level effect was significant (Table 10). Thus, it appears that total hepatic microsomal protein decreased linearly as the level of feed restriction increased (Total hepatic microsomal protein (mg) = 143.95 - 1.02 L) (Figure 12).

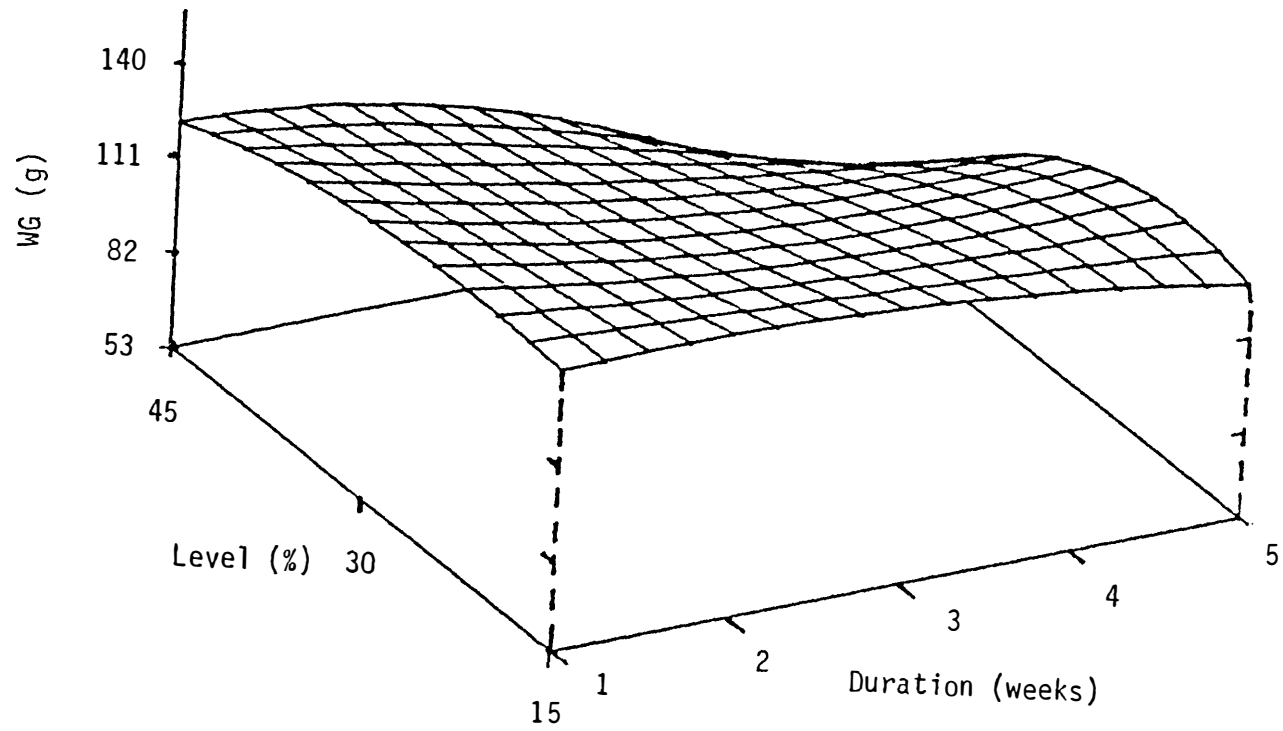


Figure 9. The effect of different levels (L) and duration (D) of feed restriction on body weight gain (WG). $WG = 63.60 + 74.28\ D + 6.52\ L - 6.16\ DL - 14.34\ D^2 + 1.17\ D^2L - 0.12\ L^2 + 0.10\ DL^2 - 0.02\ D^2L^2$ ($P \leq 0.05$).

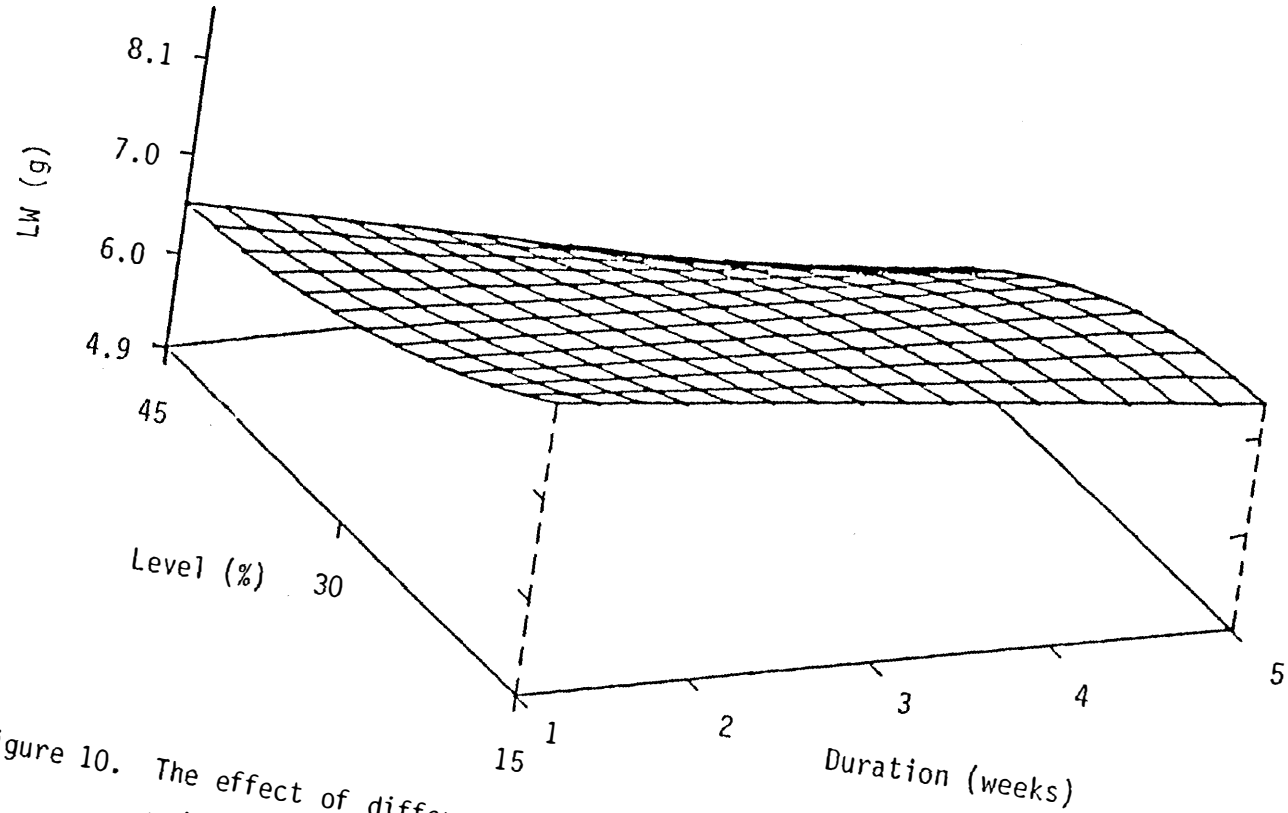


Figure 10. The effect of different levels (L) and duration (D) of feed restriction on liver weight (LW). $LW = 10.59 - 0.76\ D - 0.19\ L + 0.05\ DL + 0.002\ L^2 - 0.001\ DL^2$ ($P \leq 0.05$).

TABLE 8

EFFECT OF DIFFERENT LEVELS AND DURATION OF FEED RESTRICTION ON HEPATIC MICROSOMAL PROTEIN[1]

Level of restriction	Duration of restriction (weeks)					
	0	1	2	3	4	5
	(mg/g liver)					
0%	15.61 ± 0.47[2]					
15%		15.90 ± 0.90	18.27 ± 0.90	15.00 ± 0.90	17.50 ± 0.90	15.76 ± 0.90
30%		16.72 ± 0.81[3]	16.51 ± 0.90	16.16 ± 1.00[4]	17.32 ± 0.90	17.64 ± 0.90
45%		16.69 ± 0.90	17.19 ± 0.90	17.36 ± 0.90	17.09 ± 0.90	18.04 ± 0.90

[1]Unless otherwise stated, each value is the LSMEAN ± SEM of 5 animals.

[2]n = 17 animals.

[3]n = 6 animals.

[4]n = 4 animals.

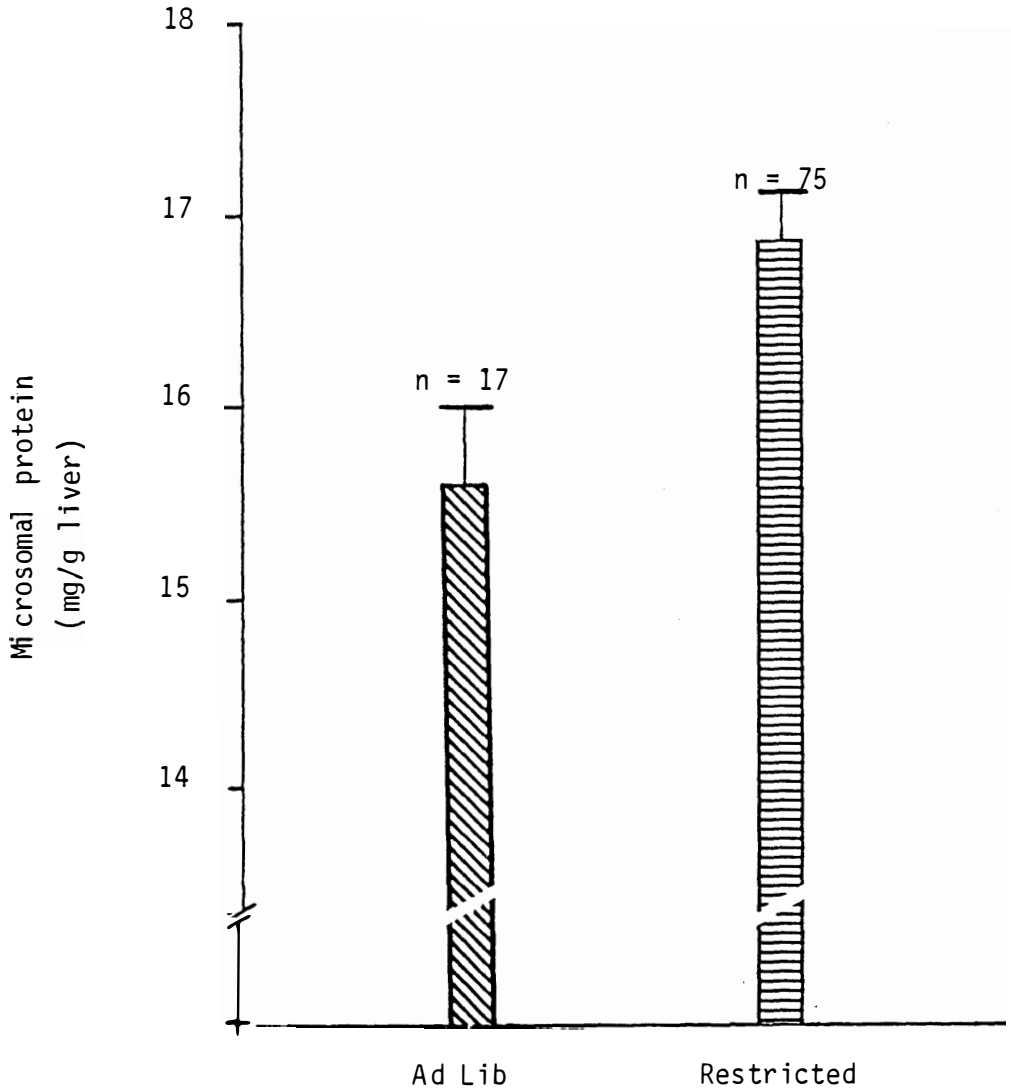


Figure 11. Hepatic microsomal protein content of ad libitum vs feed restricted rats. Values are mean + SEM and are significantly different at $P \leq 0.05$.

TABLE 9

EFFECT OF DIFFERENT LEVELS AND DURATION OF FEED RESTRICTION ON TOTAL HEPATIC MICROSOMAL PROTEIN[1]

Level of restriction	Duration of restriction (weeks)					
	0	1	2	3	4	5
	(mg)					
0%	160.21 ± 4.15[2]					
15%		130.63 ± 7.86	141.99 ± 7.85	113.14 ± 7.88	135.08 ± 7.87	114.62 ± 7.88
30%		114.19 ± 7.13[3]	116.78 ± 7.84	110.92 ± 8.85[4]	116.20 ± 7.85	117.97 ± 7.87
45%		102.59 ± 7.85	109.24 ± 7.88	103.88 ± 7.87	87.35 ± 7.86	86.48 ± 7.87

[1]Unless otherwise stated, each value is the LSMEAN ± SEM of 5 animals.

[2]n = 17 animals.

[3]n = 6 animals.

[4]n = 4 animals.

TABLE 10

F VALUE FROM THE ANALYSIS OF VARIANCE FOR THE EFFECTS OF DIFFERENT LEVELS AND DURATION OF FEED RESTRICTION ON HEPATIC CYTOCHROME P-450 (P450), TOTAL MICROSOMAL AND 100,000 X g SUPERNATANT (SN) PROTEINS

Source of variation	df	F Value		
		Microsomal Protein	100,000 x g SN Protein	P450
BLOCKS	9	1.29	5.02[1]	4.63[1]
DURATION				
linear	1	3.89	13.83[1]	0.68
quadratic	1	0.45	0.03	0.00
cubic	1	0.30	0.67	0.30
LEVEL				
linear	1	34.70[1]	66.71[1]	7.96[1]
quadratic	1	0.55	1.38	0.44
DURATION X LEVEL INTERACTIONS				
Duration x level	1	0.37	4.63[1]	0.02
Duration^2 x level	1	0.16	0.94	0.07
Duration x level^2	1	2.66	0.13	0.28
Duration^2 x level^2	1	0.32	0.07	1.99
Duration^3 x level	1	1.10	4.34[1]	0.43
Duration^3 x level^2	1	0.13	1.83	1.72

[1] $P \leq 0.05$.

[2] Quadratic function.

[3] Cubic function.

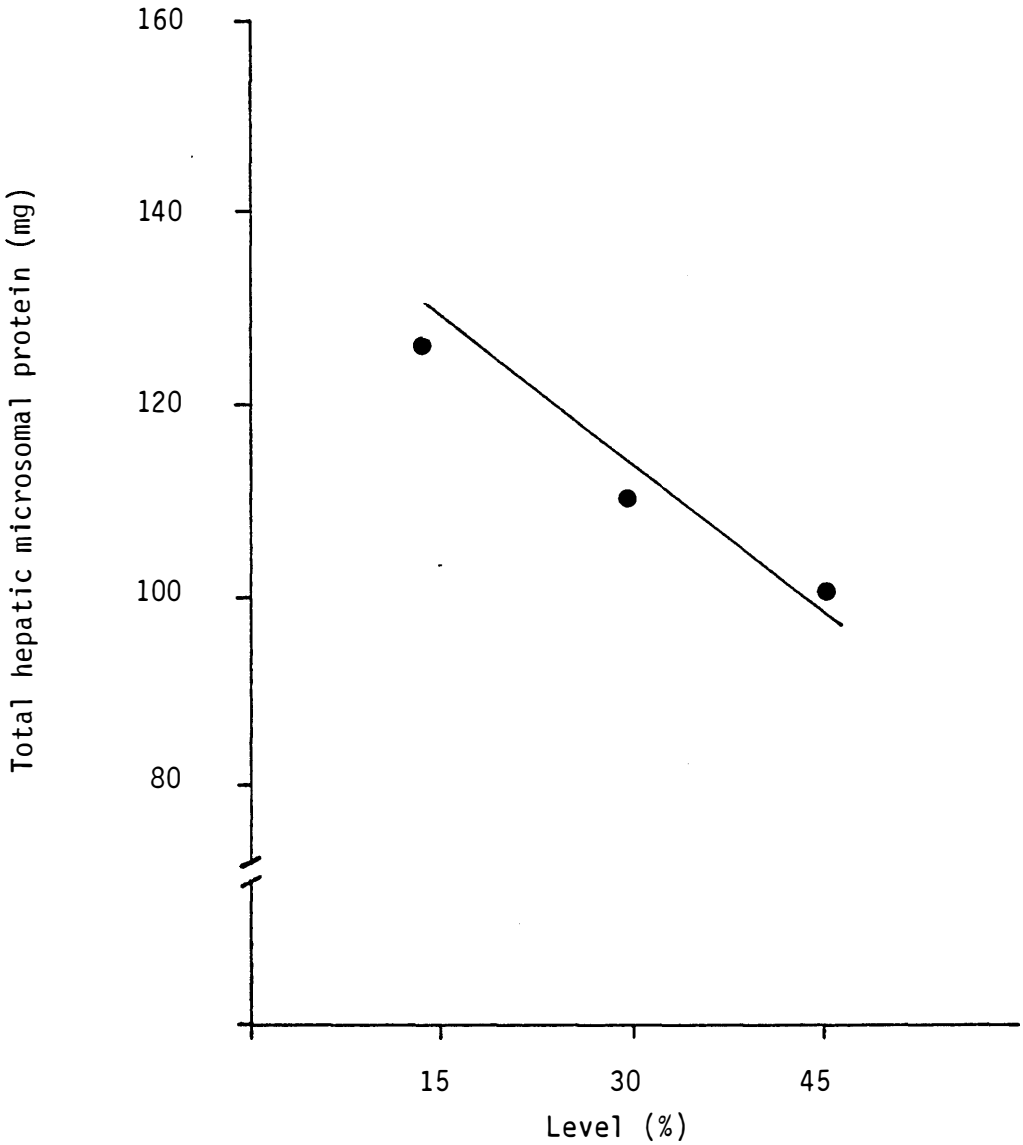


Figure 12. The effect of different levels (L) of feed restriction on total hepatic microsomal protein (TMP). TMP = 143.95 - 1.02 L ($P \leq 0.05$). ● n = 25.

The hepatic protein content of 100,000 x g SN (expressed as per gram of wet liver tissue) remained basically unaltered by feed restriction (Table 11). However, when this was expressed as the total hepatic protein content, a general pattern of decreasing hepatic 100,000 x g SN protein evolved with increasing levels and duration of feed restriction (Table 12). This decrease was significantly affected by the level as well as the duration of feed restriction (Table 10). The response surface included a significant cubic function of the duration effect and at the lower level of feed restriction there was a lack of a consistent pattern for the changes (Figure 13)(Total 100,000 x g SN (mg) = 1380.14 - 760.64 D - 20.67 L + 21.30 DL + 277.77 D^2 - 8.00 D^2L - 29.58 D^3 + 0.84 D^3L).

The hepatic cytochrome P-450 content (nmoles/g liver) increasd with increasing levels and duration of feed restriction (Table 13). However, only the effect of the level of restriction was statistically significant (Table 10). The increase in the hepatic cytochrome P-450 content was linear with the increase in the level of feed restriction as shown in Figure 14 (Hepatic cytochrome P-450 content (nmoles/g liver) = 14.36 + 0.05 L).

The in vitro hepatic NADPH cytochrome c reductase activity (μmoles cytochrome c reduced/min/g liver), on the other hand, remained unaltered by the effects of different levels and duration of feed restriction (Table 14). However, the mean activity of the hepatic cytochrome c reductase in the feed restricted group (taken together) was significantly lower than that in the ad libitum group

TABLE 11

EFFECT OF DIFFERENT LEVELS AND DURATION OF FEED RESTRICTION ON HEPATIC 100,000 x g SUPERNATANT (SN) PROTEIN[1]

Level of restriction	Duration of restriction (weeks)					
	0	1	2	3	4	5
	(mg/g liver)					
0%	90.04 ± 2.13[2]					
15%		91.88 ± 4.02	88.79 ± 4.02	89.77 ± 4.03	98.98 ± 4.03	92.08 ± 4.03
30%		97.58 ± 3.65[3]	94.73 ± 4.01	89.39 ± 4.53[4]	90.84 ± 4.01	89.28 ± 4.03
45%		94.09 ± 4.02	89.57 ± 4.03	89.09 ± 4.03	90.61 ± 4.02	88.45 ± 4.03

[1]Unless otherwise stated, each value is the LSMEAN ± SEM of 5 animals.

[2]n = 17 animals.

[3]n = 6 animals.

[4]n = 4 animals.

TABLE 12

EFFECT OF DIFFERENT LEVELS AND DURATION OF FEED RESTRICTION ON TOTAL 100,000 x g SUPERNATANT (SN) PROTEIN[1]

Level of restriction	Duration of restriction (weeks)					
	0	1	2	3	4	5
	(mg)					
0%	924.66 ± 20.92[2]					
15%		762.57 ± 39.56	690.35 ± 39.54	682.00 ± 39.67	766.64 ± 39.66	670.38 ± 39.67
30%		667.21 ± 35.89[3]	668.71 ± 39.51	618.15 ± 44.55[4]	604.31 ± 39.51	605.16 ± 39.62
45%		573.42 ± 39.54	570.69 ± 39.67	525.20 ± 39.66	470.10 ± 39.56	414.05 ± 39.66

[1]Unless otherwise stated, each value is the LSMEAN ± SEM of 5 animals.

[2]n = 17 animals.

[3]n = 6 animals.

[4]n = 4 animals.

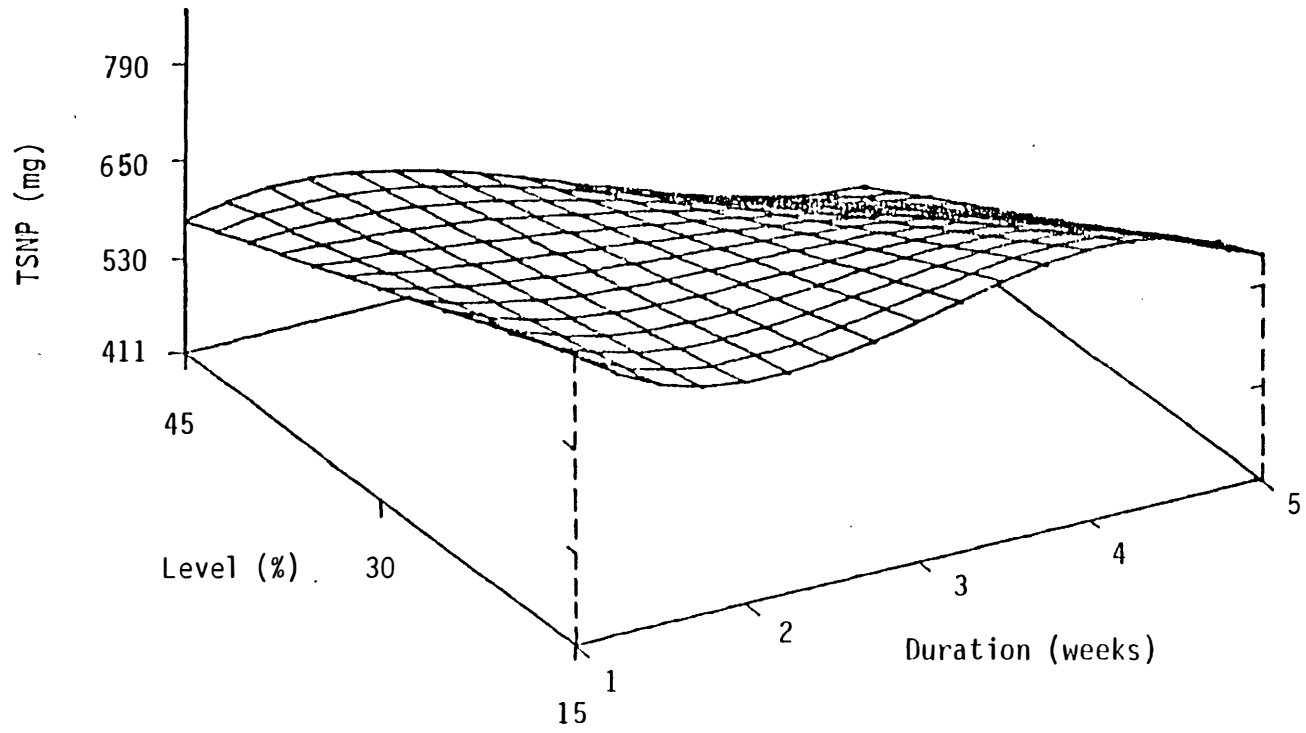


Figure 13. The effect of different levels (L) and duration (D) of feed restriction on total 100,000 x g supernatant proteins (TSNP). TSNP = 1380.14 - 760.64 D - 20.67 L + 21.30 DL + 277.77 D^2 - 8.00 D^2L - 29.58 D^3 + 0.84 D^3L ($P \leq 0.05$).

TABLE 13

EFFECT OF DIFFERENT LEVELS AND DURATION OF FEED RESTRICTION ON HEPATIC CYTOCHROME P-450 CONTENT[1]

Level of restriction	Duration of restriction (weeks)					
	0	1	2	3	4	5
	(nmoles/g liver)					
0%	13.4 ± 0.43[2]					
15%		14.1 ± 0.82	16.1 ± 0.81	14.3 ± 0.82	15.6 ± 0.82	15.1 ± 0.82
30%		16.8 ± 0.74[3]	15.4 ± 0.81	15.2 ± 0.92[4]	16.9 ± 0.92[4]	16.3 ± 0.82
45%		16.1 ± 0.81	16.7 ± 0.82	16.8 ± 82	17.4 ± 0.82	16.6 ± 0.82

[1] Unless otherwise stated, each value is the LSMEAN ± SEM of 5 animals.

[2] n = 17 animals.

[3] n = 6 animals.

[4] n = 4 animals.

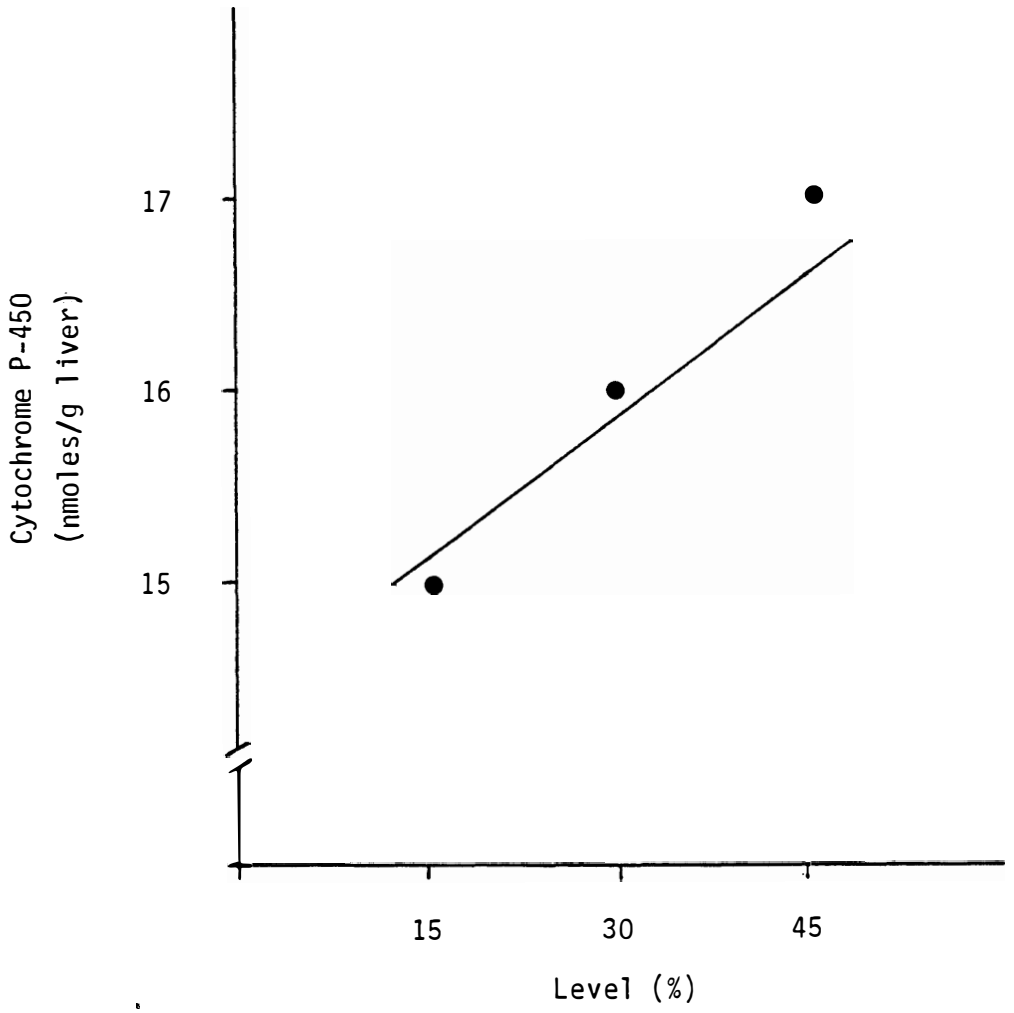


Figure 14. The effect of different levels (L) of feed restriction on hepatic cytochrome P-450 content (450). P-450 = 14.36 + 0.05 L ($P \leq 0.05$). ● n = 25.

TABLE 14

EFFECT OF DIFFERENT LEVELS AND DURATION OF FEED RESTRICTION ON THE IN VITRO ACTIVITIES OF HEPATIC NADPH CYTOCHROME C REDUCTASE[1]

Level of restriction	Duration of restriction (weeks)					
	0	1	2	3	4	5
	(μmoles cytochrome c reduced/min/g liver)					
0%	3.56 ± 0.13[2]					
15%		2.66 ± 0.24	2.88 ± 0.24	2.99 ± 0.24	2.77 ± 0.24	2.73 ± 0.24
30%		2.68 ± 0.22[3]	2.89 ± 0.24	2.45 ± 0.27[4]	2.75 ± 0.24	2.96 ± 0.24
45%		2.54 ± 0.24	2.83 ± 0.24	2.76 ± 0.24	2.88 ± 0.24	3.30 ± 0.24

[1] Unless otherwise stated, each value is the LSMEAN ± SEM of 5 animals.

[2] n = 17 animals.

[3] n = 6 animals.

[4] n = 4 animals.

(Figure 15) (2.8 $\pm$ 0.06 vs 3.6 $\pm$ 0.15 μmoles of cytochrome c reduced/min/g liver, respectively).

The in vitro activities of hepatic aniline hydroxylase increased with increasing levels and duration of feed restriction (Table 15). Statistical analysis of the data revealed that this increase was linear with the increase in both parameters of restriction (Table 16). This linear response surface is presented in Figure 16 and the highest effect on the in vitro enzymatic activity was observed at 45% feed restriction for five weeks (aniline hydroxylase activity (nmoles of HCHO formed/min/g liver) = 5.90 + 0.81 D + 0.14 L).

The in vitro activities of hepatic PCMA-N-demethylase (nmoles of PCA formed/min/g liver) and p-nitrophenol UDP-glucuronyl transferase (nmoles of p-nitrophenol conjugated/min/g liver) are presented in Tables 17 and 18, respectively. Feed restriction did not alter significantly the in vitro activities of these two enzymes when expressed as per gram of wet liver tissue (Table 16).

The in vitro activity of hepatic glucose-6-phosphate dehydrogenase increased with increasing levels and duration of feed restriction (Table 19). The highest in vitro activity of hepatic glucose-6-phosphate dehydrogenase was observed at the 45% restriction for five weeks. However, only the duration of feed restriction was found to be statistically significant (Table 20). This significant increase in glucose-6-phosphate dehydrogenase activity over the increase in duration of feed restriction was linear and is shown in

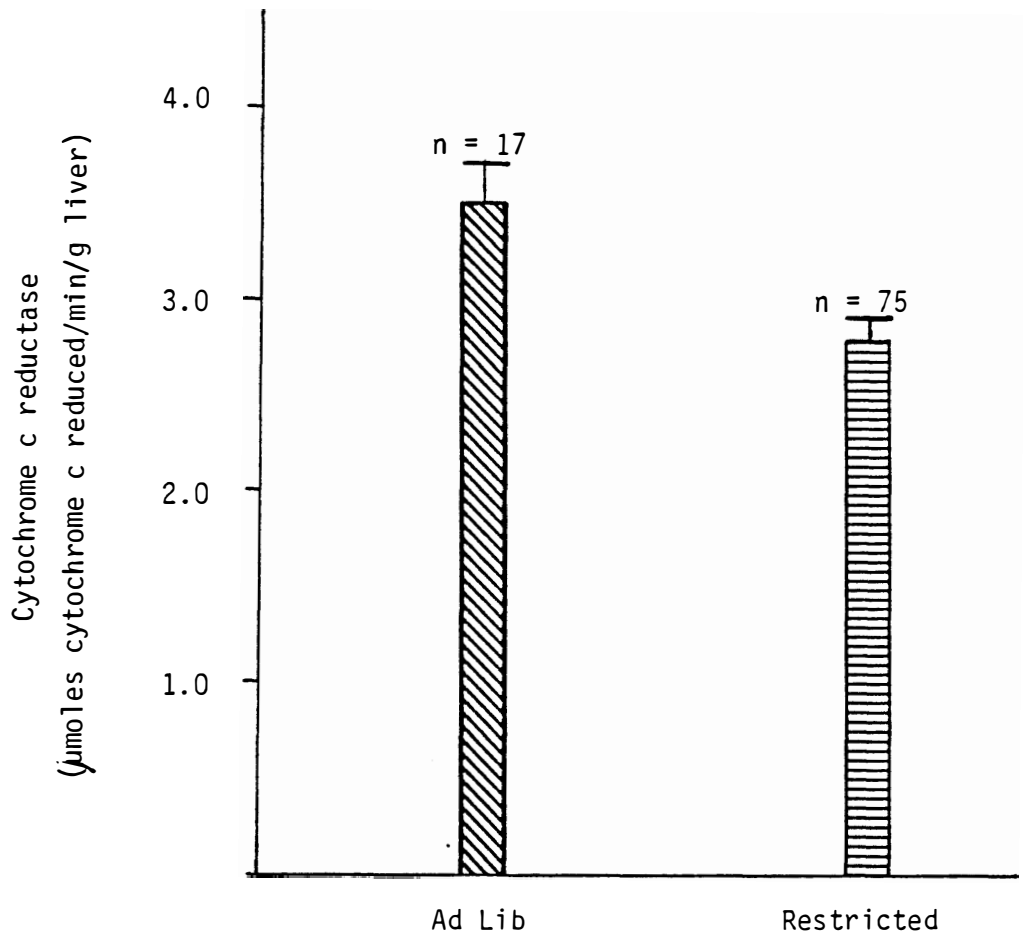


Figure 15. Hepatic cytochrome c reductase activity of ad libitum vs feed restricted rats. Values are mean ± SEM and are significantly different at $P \leq 0.05$.

TABLE 15

EFFECT OF DIFFERENT LEVELS AND DURATION OF FEED RESTRICTION ON THE IN VITRO ACTIVITIES OF HEPATIC ANILINE HYDROXYLASE[1]

Level of restriction	Duration of restriction (weeks)					
	0	1	2	3	4	5
	(nmoles HCHO formed/min/g liver)					
0%	10.1 ± 0.47[2]					
15%		8.7 ± 0.89	10.3 ± 0.89	8.6 ± 0.90	12.8 ± 0.89	11.2 ± 0.90
30%		11.7 ± 0.81[3]	12.5 ± 0.89	11.8 ± 1.01[4]	14.0 ± 0.89	14.3 ± 0.89
45%		12.3 ± 0.89	13.4 ± 0.90	14.2 ± 0.89	14.8 ± 0.89	16.3 ± 0.89

[1] Unless otherwise stated, each value is the LSMEAN ± SEM of 5 animals.

[2] n = 17 animals.

[3] n = 6 animals.

[4] n = 4 animals.

TABLE 16

F VALUE FROM THE ANALYSIS OF VARIANCE FOR THE EFFECTS OF DIFFERENT LEVELS AND DURATION OF FEED RESTRICTION ON THE IN VITRO ACTIVITIES OF HEPATIC ANILINE HYDROXYLASE (AH), PCMA-N-DEMETHYLASE (PCMAD) AND UDP-GLUCURONYL TRANSFERASE (GT)

Source variation	df	F Value		
		AH	PCMAD	GT
BLOCKS	9	2.94[1]	5.24[1]	4.90[1]
DURATION				
linear	1	20.13[1]	0.30	0.18
quadratic	1	0.10	0.00	0.51
cubic	1	0.09	1.04	0.33
LEVEL				
linear	1	34.74[1]	0.38	0.02
quadratic	1	1.73	0.02	0.00
DURATION x LEVEL INTERACTIONS				
Duration x level	1	0.30	0.13	1.47
Duration^2 x level	1	0.01	0.16	0.05
Duration x level^2	1	0.10	1.05	1.42
Duration^2 x level^2	1	0.04	0.98	0.53
Duration^3 x level	1	0.88	0.80	0.07
Duration^3 x level^2	1	0.00	1.14	0.00

[1] $P \leq 0.05$.

[2] Quadratic function.

[3] Cubic function.

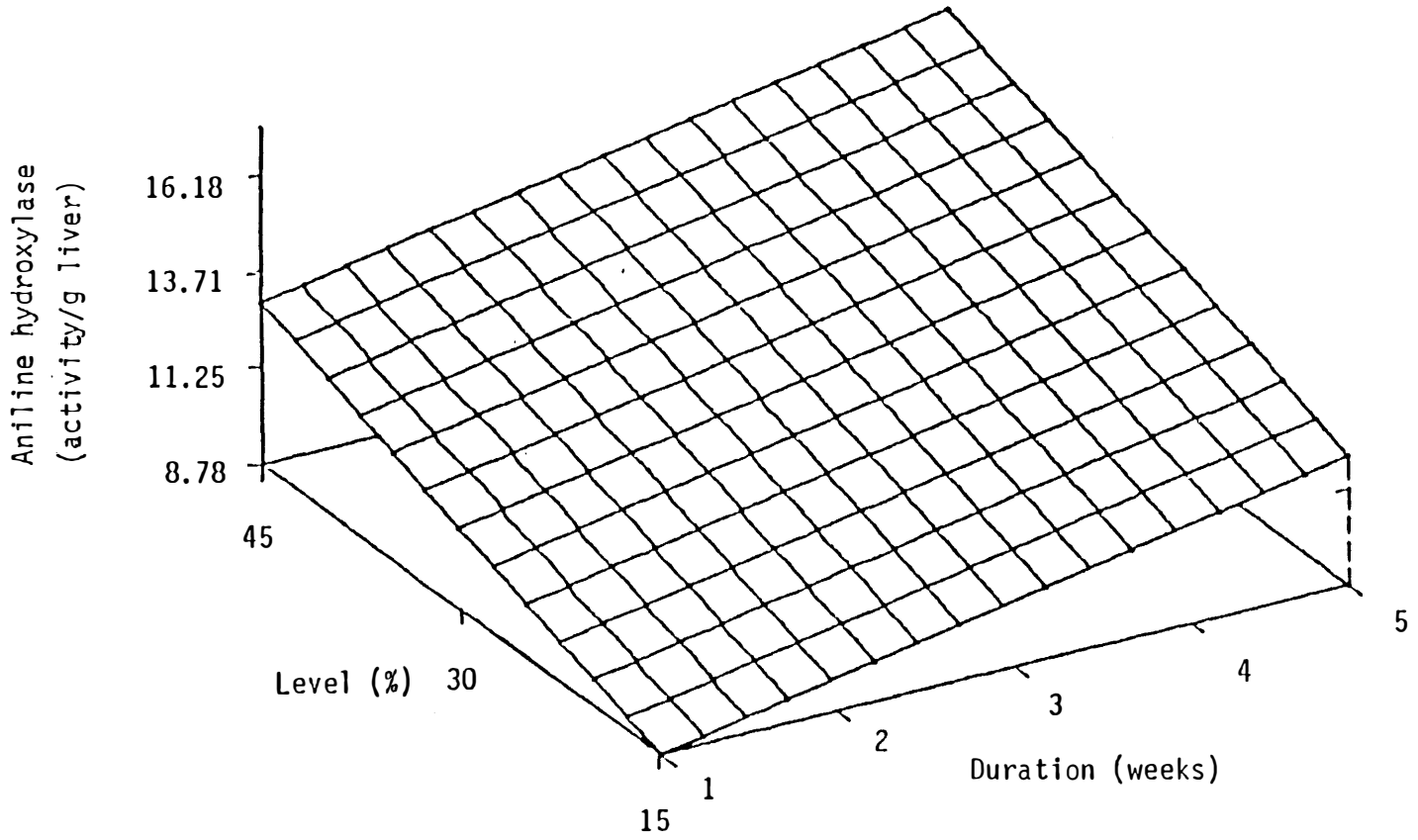


Figure 16. The effect of different levels (L) and duration (D) of feed restriction on the in vitro activities of hepatic aniline hydroxylase (AH). AH = 5.90 + 0.81 D + 0.14 L ($P \leq 0.05$). Activity = nmoles HCHO formed/min.

TABLE 17

EFFECT OF DIFFERENT LEVELS AND DURATION OF FEED RESTRICTION ON THE IN VITRO ACTIVITIES OF HEPATIC PCMA-N-DEMETHYLASE[1]

Level of restriction	Duration of restriction (weeks)					
	0	1	2	3	4	5
	(nmoles PCA formed/min/g liver)					
0%	22.7 ± 1.4[2]					
15%		20.7 ± 2.7	21.0 ± 2.7	27.8 ± 2.7	19.7 ± 2.7	23.8 ± 2.7
30%		23.0 ± 2.5[3]	24.5 ± 2.7	20.3 ± 3.1[4]	18.8 ± 2.7	22.7 ± 2.7
45%		19.7 ± 2.7	18.9 ± 2.7	21.4 ± 2.7	22.3 ± 2.7	21.8 ± 2.7

[1] Unless otherwise stated, each value is the LSMEAN ± SEM of 5 animals.

[2] n = 17 animals.

[3] n = 6 animals.

[4] n = 4 animals.

TABLE 18

EFFECT OF DIFFERENT LEVELS AND DURATION OF FEED RESTRICTION ON THE IN VITRO ACTIVITIES OF HEPATIC UDP-GLUCURONYL TRANSFERASE[1]

Level of restriction	Duration of restriction (weeks)					
	0	1	2	3	4	5
	(nmoles p-nitrophenol conjugated/min/g liver)					
0%	55.60 ± 3.51[2]					
15%		55.31 ± 6.37	62.79 ± 6.38	45.17 ± 6.40	52.17 ± 6.40	44.44 ± 6.40
30%		45.04 ± 5.79[3]	52.77 ± 6.37	55.92 ± 7.19[4]	51.71 ± 6.37	53.21 ± 8.39
45%		49.01 ± 6.38	50.82 ± 6.40	50.49 ± 6.40	50.26 ± 7.20[4]	51.53 ± 6.40

[1]Unless otherwise stated, each value is the LSMEAN ± SEM of 5 animals.

[2]n = 16 animals.

[3]n = 6 animals.

[4]n = 4 animals.

TABLE 19

EFFECT OF DIFFERENT LEVELS AND DURATION OF FEED RESTRICTION ON THE IN VITRO ACTIVITIES OF HEPATIC GLUCOSE-6-PHOSPHATE DEHYDROGENASE[1]

Levels of restriction	Duration of restriction (weeks)					
	0	1	2	3	4	5
	(μmoles NADPH formed/min/g liver)					
0%	1.10 ± 0.21[2]					
15%		1.09 ± 0.40	2.43 ± 0.40	1.35 ± 0.40	2.79 ± 0.40	2.35 ± 0.40
30%		1.71 ± 0.36[3]	2.49 ± 0.40	2.07 ± 0.45[4]	2.78 ± 0.40	2.50 ± 0.40
45%		1.68 ± 0.40	1.80 ± 0.40	2.12 ± 0.40	3.47 ± 0.40	3.12 ± 0.40

[1] Unless otherwise stated, each value is the LSMEAN ± SEM of 5 animals.

[2] n = 17 animals.

[3] n = 6 animals.

[4] n = 4 animals.

TABLE 20

F VALUE FROM THE ANALYSIS OF VARIANCE FOR THE EFFECTS OF DIFFERENT LEVELS AND DURATION OF FEED RESTRICTION ON THE IN VITRO ACTIVITIES OF HEPATIC GLUCOSE-6-PHOSPHATE DEHYDROXYGENASE (G6PD), 6-PHOSPHOGLUCONATE DEHYDROGENASE (6PGD) AND MALIC ENZYME (ME)

Source of variation	df	F Value		
		G6PD	6PGD	ME
BLOCKS	9	1.32	6.68[1]	3.77[1]
DURATION				
linear	1	15.04[1]	0.28	14.21[1]
quadratic	1	0.37	1.84	0.58
cubic	1	0.22	0.91	0.68
LEVEL				
linear	1	1.97	8.54[1]	0.06
quadratic	1	0.13	1.54	4.67[1]
DURATION x LEVEL INTERACTIONS				
Duration x level	1	0.86	0.05	0.03
Duration^2 x level	1	0.18	0.25	1.00
Duration x level^2	1	0.95	10.80[1]	1.74
Duration^2 x level^2	1	0.39	0.01	0.07
Duration^3 x level	1	1.46	0.03	0.00
Duration^3 x level^2	1	0.29	0.19	1.10

[1] $P \leq 0.05$.

[2] Quadratic function.

[3] Cubic function.

Figure 17 (Glucose-6-phosphate dehydrogenase activity (μmoles NADPH formed/min/g liver) = 1.32 + 0.31 D).

The data for the in vitro activity of hepatic 6-phosphogluconate dehydrogenase (μmoles NADPH formed/min/g liver) is presented in Table 21. The activity was altered by the level as well as duration of feed restriction. At the 15% and 45% levels of feed restriction, the 6-phosphogluconate dehydrogenase activity increased with increasing duration of feed restriction while at the 30% level, the activity decreased with increasing duration of feed restriction. Concurrently, the activity also decreased with increasing levels of feed restriction in a curvilinear manner. This is represented by the response surface in Figure 18 (6-phosphogluconate dehydrogenase activity (μmoles of NADPH formed/min/g liver) = 1.204 + 0.873 D + 0.220 L - 0.067 DL - 0.00388 L^2 + 0.00111 DL^2).

The in vitro activity of hepatic malic enzyme (μmoles NADPH formed/min/g liver) also increased with increasing levels and duration of feed restriction (Table 22). This increase was significantly linear and curvilinear with increasing duration, and levels of feed restriction, respectively (Table 20). The response is presented in Figure 19 and the duration of feed restriction exerted a greater effect on the enzyme activity than the level of feed restriction (hepatic malic enzyme activity (μmoles NADPH formed/min/g liver) = 0.258 + 0.103 D + 0.047 L - 0.0008 L^2).

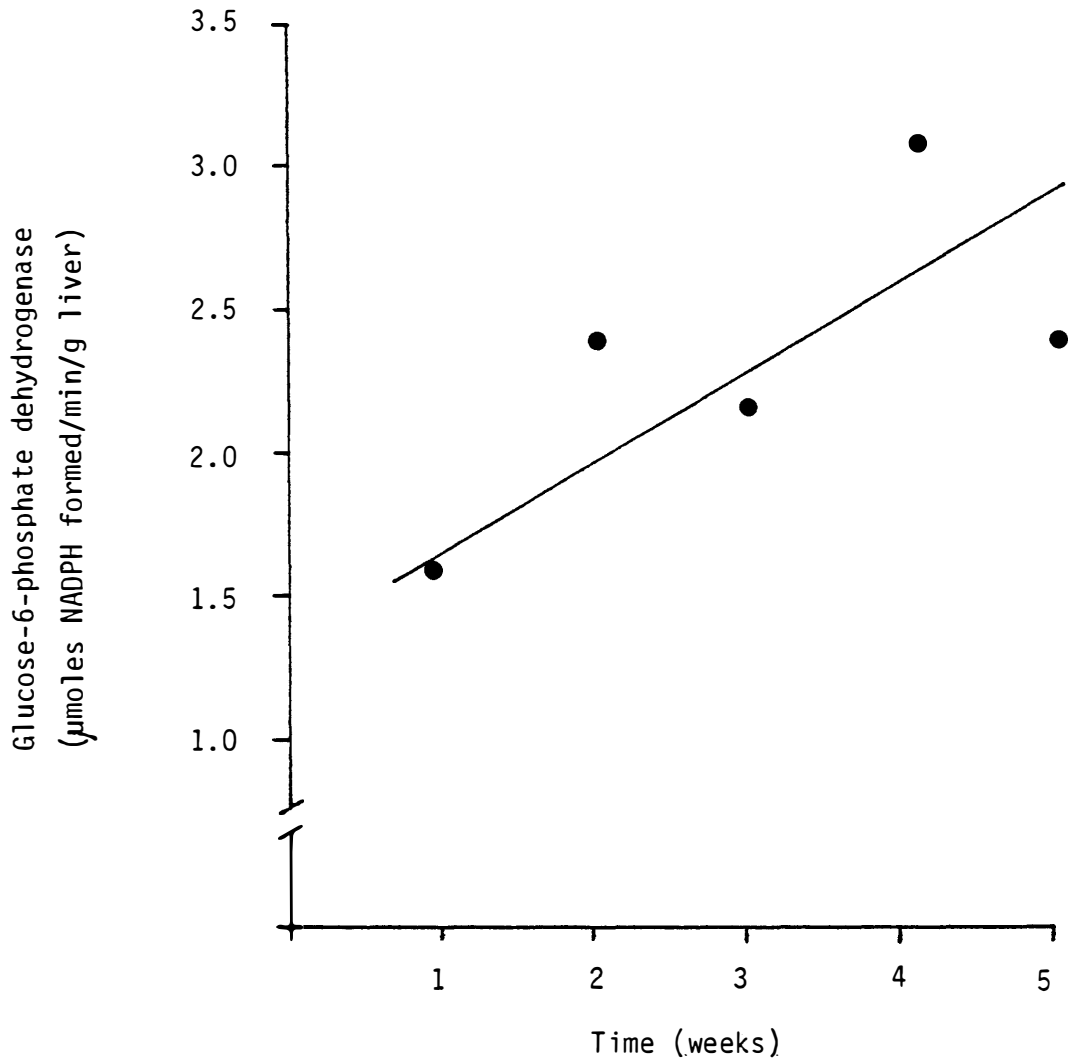


Figure 17. The effect of the duration (D) of feed restriction on glucose-6-phosphate dehydrogenase (G6PD). G6PD = 1.32 + 0.31 D ($P \leq 0.05$). ●

TABLE 21

EFFECT OF DIFFERENT LEVELS AND DURATION OF FEED RESTRICTION ON THE IN VITRO ACTIVITIES OF HEPATIC 6-PHOSPHOGLUCONATE DEHYDROGENASE[1]

Level of restriction	Duration of restriction (weeks)					
	0	1	2	3	4	5
	(μmoles NADPH formed/min/g liver)					
0%	4.05 ± 0.11[2]					
15%		3.76 ± 0.20	3.86 ± 0.20	3.78 ± 0.21	4.23 ± 0.21	4.14 ± 0.21
30%		4.25 ± 0.19[3]	4.09 ± 0.20	3.57 ± 0.23[4]	3.80 ± 0.20	3.59 ± 0.20
45%		3.50 ± 0.20	3.30 ± 0.21	3.42 ± 0.21	3.68 ± 0.20	3.75 ± 0.21

[1] Unless otherwise stated, each value is the LSMEAN ± SEM of 5 animals.

[2] n = 17 animals.

[3] n = 6 animals.

[4] n = 4 animals.

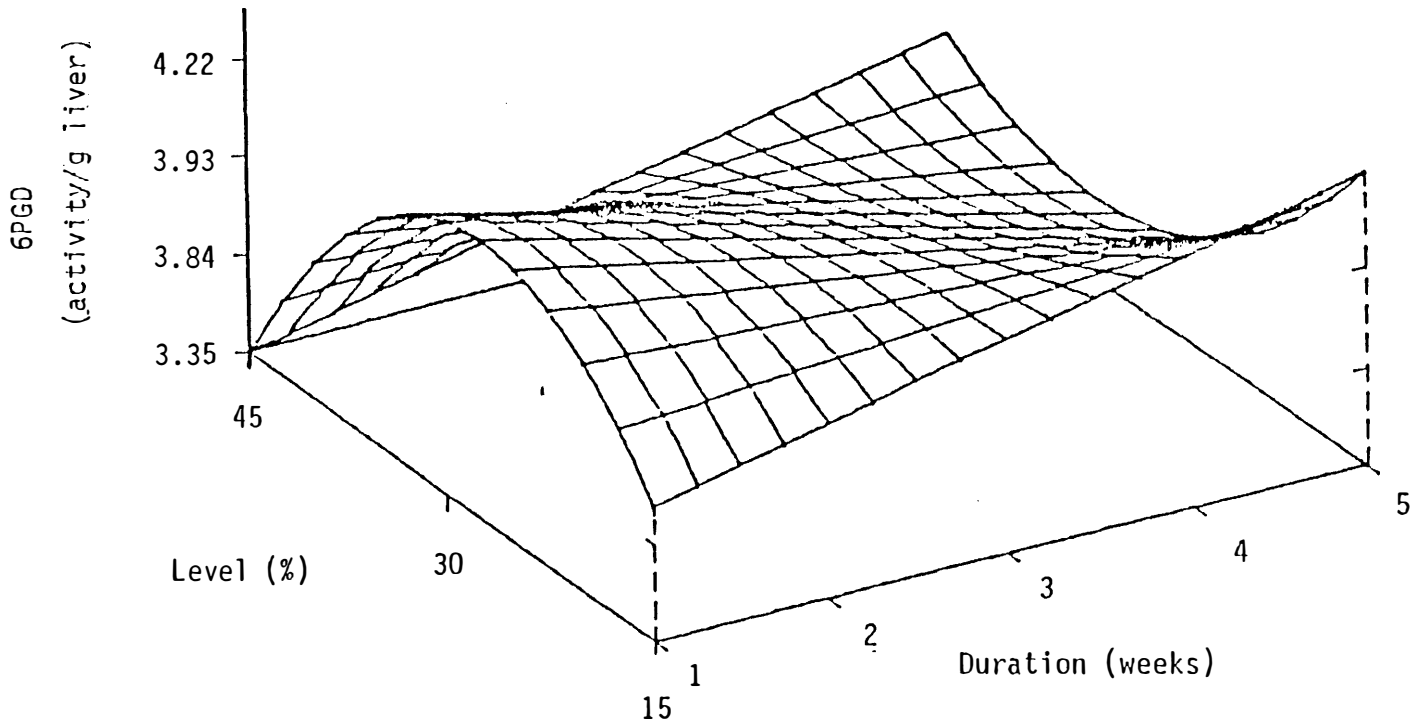


Figure 18. The effect of different levels (L) and duration (D) of feed restriction on the in vitro activities of hepatic 6-phosphogluconate dehydrogenase (6PGD). $6PGD = 1.204 + 0.873\ D + 0.220\ L - 0.067\ DL - 0.004\ L^2 + 0.001\ DL^2$ ($P \leq 0.05$). Activity = μmoles of NADPH formed/min.

TABLE 22

EFFECT OF DIFFERENT LEVELS AND DURATION OF FEED RESTRICTION ON THE IN VITRO ACTIVITIES OF HEPATIC MALIC ENZYME[1]

Level of restriction	Duration of restriction (weeks)					
	0	1	2	3	4	5
	(μmoles NADPH formed/min/g liver)					
0%	0.91 ± 0.07[2]					
15%		0.73 ± 0.14	1.14 ± 0.14	0.87 ± 0.14	1.40 ± 0.14	1.27 ± 0.14
30%		1.13 ± 0.13[3]	1.40 ± 0.14	1.05 ± 0.16[4]	1.23 ± 0.14	1.45 ± 0.14
45%		0.90 ± 0.14	0.92 ± 0.14	1.04 ± 0.14	1.14 ± 0.14	1.41 ± 0.14

[1] Unless otherwise stated, each value in the LSMEAN ± SEM of 5 animals.

[2] n = 17 animals.

[3] n = 6 animals.

[4] n = 4 animals.

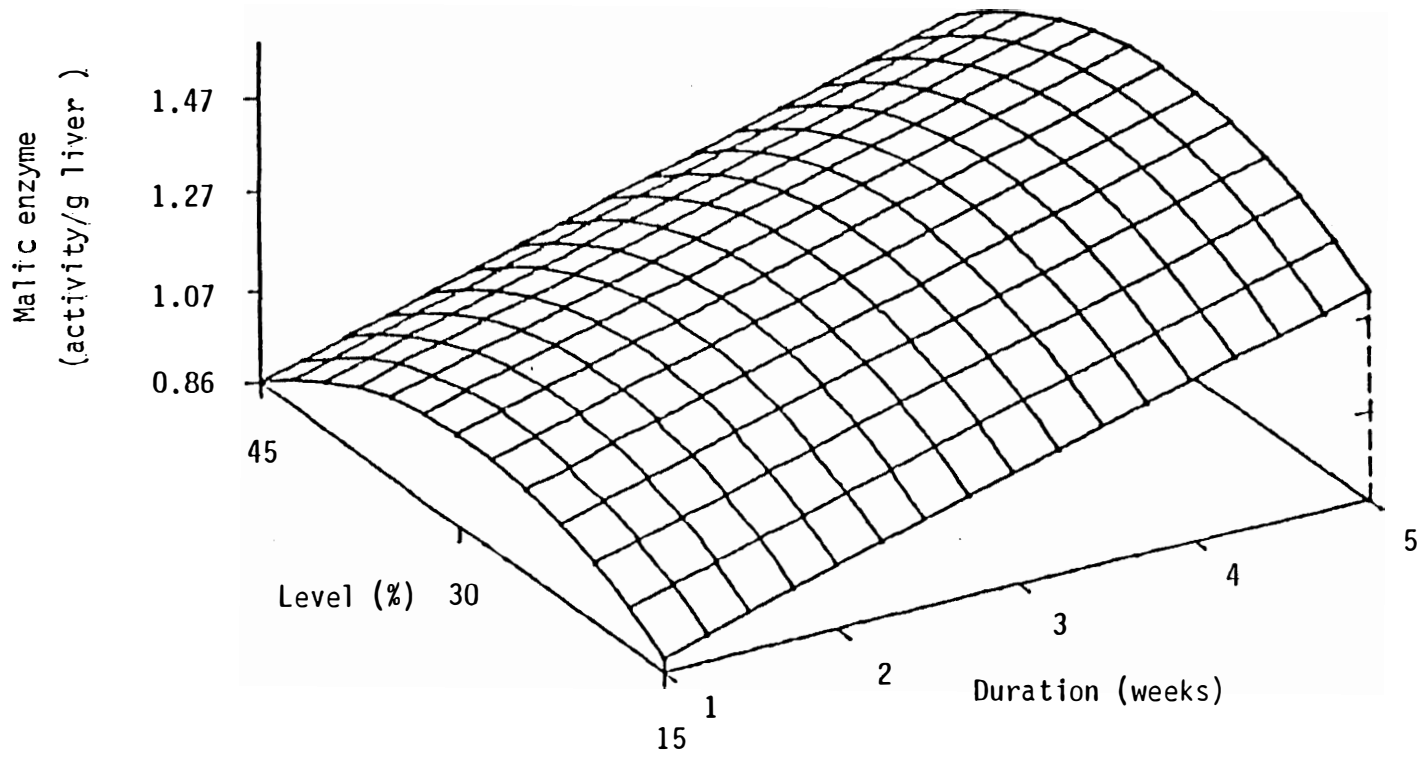


Figure 19. The effect of different levels (L) and duration (D) of feed restriction on the in vitro activities of hepatic malic enzyme (ME). ME = 0.258 + 0.103 D + 0.047 L - 0.0008 L^2 ($P \leq 0.05$). Activity = μmoles NADPH formed/min.

2. EXPERIMENT II

This study was carried out to further substantiate the effects of feed restriction on the activities of the hepatic enzymes examined in Experiment I by determining the in vivo metabolism of a drug that is metabolized by the mixed function oxidase system. From the results in Experiment I, the changes in the in vitro activities of the enzymes examined were not following any consistent patterns at the lower levels and duration of feed restriction. The effect of feed restriction on these enzymes was most marked after four weeks of the 45% level of restriction.

In this experiment, the treatment group of rats were thus feed restricted at 45% for four weeks before being dosed with the antipyrine (40 mg/kg body weight). The body weight and feed consumption data are presented in Table 23. The feed restricted group of animals had lower body weights (35%) and weight gain (52%) than the ad libitum animals. As shown in Table 24, the apparent volume of distribution of antipyrine in the 45% feed restricted rats was approximately 46% less when compared to the ad libitum rats. The blood clearance was 39% less while the blood half life was 11% less in the 45% feed restricted rats when compared to the ad libitum controls. All the decreases in these parameters are significant at $P \leq 0.05$.

3. EXPERIMENT III

In this experiment, the animals were the same animals that were used for the antipyrine experiment. They were allowed to recover

TABLE 23

EFFECT OF 45% FEED RESTRICTION FOR FOUR WEEKS ON FINAL BODY WEIGHTS[1] AND FEED CONSUMPTION[1]

Parameter	Ad libitum	45% restricted
Initial body weight (g)	117.2 ± 1.60	121.3 ± 1.91
Final body weight (g)	271.9 ± 4.81	195.3 ± 2.55[2]
Weight gain (g)	154.7 ± 4.01	74.0 ± 3.02[2]
Feed consumption (g)	444.5 ± 49.94	279.3 ± 4.63[2]

[1]Each value is the mean ± SEM of 9 rats per group.

[2]Statistically significant at $P \leq 0.05$.

TABLE 24

EFFECT OF 45% FEED RESTRICTION FOR FOUR WEEKS ON THE METABOLISM OF ANTIPYRINE

Parameter	ad libitum	45% restricted
Volume of distribution (ml)	144.64 + 14.90[1]	78.21 + 3.49[2]
Blood clearance (ml/min)	1.92 + 0.24	1.17 + 0.09[2]
Blood half life (min)	53.00 + 1.75	47.22 + 1.91[2]

[1]Values are mean ± SEM. of 9 rats per group.

[2]Significantly different from the ad lib group ($P \leq 0.05$).

from any possible effects of the antipyrine study for approximately 36 - 42 days before the toxicity of the LD_{50} (5.4 mg/kg body weight) dose of carbon tetrachloride was examined. The data on mortality, body weight and liver weight are presented in Table 25. As expected the 45% feed restricted rats had significantly lower mean body weights at the time of drug administration and at death. The mean liver weight was also lower although this is not statistically significant. However, the liver as a percentage of body weight at death was significantly higher. The mortality at the 5.4 mg/kg dose of carbon tetrachloride were seven out of ten and four out of five for the ad libitum and 45% feed restricted groups respectively. At the end of one week after carbon tetrachloride administration, the three surviving rats in the ad libitum group had resumed feeding and did not show any morbidity. The one surviving rat in the 45% feed restricted group on the other hand was still off feed,and the animal had a rough hair coat, nasal exudates and was very lethargic.

TABLE 25

BODY WEIGHT, LIVER WEIGHT AND MORTALITY DATA OF THE AD LIBITUM VS 45% FEED RESTRICTED RATS DOSED WITH CARBON TETRACHLORIDE.

Parameter	Ad Libitum	45% restricted
No. of animals	10	5
Body weight at time of dosage	330.3 ± 9.79[1]	228.0 ± 8.92[2]
Mortality at 5.4 mg/kg body weight dose of carbon tetrachloride	7/10	4/5
Body weight at time of death	319.3 ± 8.48	207.2 ± 9.22[2]
Liver weight	13.7 ± 0.36	11.7 ± 1.04
Liver weight as % of body weight	4.3 ± 0.09	5.7 ± 0.29[2]

[1]Values are mean ± SEM.

[2]Statistically significant at $P \leq 0.05$.

CHAPTER V

DISCUSSION

Numerous studies have been published on the effects of starvation on in vivo and in vitro drug metabolism of drugs and chemicals (8-11,65-71). It is generally believed that starvation results in decreased drug metabolism and decreased activities of drug metablizing enzymes; however, much depends on the kind of substrate used. Influenced by the increasing practice of food restriction in contemporary society, the effects of feed restriction was examined in an animal model. It was found that 45% feed restriction for 28 days and 50% feed restriction for 49 days resulted in increased activities of certain drug metabolizing enzymes and this increase correlated well with the increased in vivo metabolism of hexobarbital (14,16). Thus it became important to further define the degree (level) and the duration (length of restriction period) of feed restriction needed to produce enhanced in vitro drug metabolizing enzyme activity, NADPH-generating enzyme activity and in vivo drug metabolism as well as determine the pattern of these changes.

1. GROWTH PARAMETERS

Body weight changes

Increasing levels and duration of feed restriction resulted in progressive curvilinear decreases in the amount of body weight gain and consequently the final body weight of the animals during the experimental period (Figure 8, page 68). The lower weight gain was in

direct response to the reduced feed consumption of the rats and was most marked at the higher levels and duration of feed restriction. This lower body weight gain after a period of partial feed restriction observed is consistent with findings in the literature (13-16). At 45% feed restriction for four weeks, the lower body weight gain when compared with the ad libitum rats (49% less) in the current study is very similar to that (54% less) in the study by Sachan (14). Starvation for one to three days has been also shown to progressively decrease the final body weight (9,67). However, it must be pointed out that during starvation, the decrease in weight which is rapid in the beginning is largely due to the loss of body water while tissue composition remains relatively unaltered (7). The age of the animals and/or the initial body weight are important considerations in comparing changes in the body weight. For example, in growing animals the rate of gain will be the primary effect while in the adult and older animals, it would be the loss in the body weight. Further, the percent changes will be lesser in adults and older animals because of their initial body weight. On the basis of these results, it is possible to predict the body weight gain of rats by imposing a selected level of restriction and the duration of that restriction. For example, if the desired body weight for a weanling Sprague Dawley rat at the end of four weeks was 80 g, the level of restriction imposed should be 45%.

Liver weight changes

The changes in the liver weights were related to the changes in body weights (Figure 10, page 71). The liver weight expressed as a percent of the final body weight for the ad libitum group and the 45% feed restricted for 4 weeks (3.9% and 2.8% respectively) was very similar to that (4.2% and 2.9% respectively) by Sachan (14). Liver weight has also been reported to decrease progressively with the duration of starvation (9,67,92). For example, the liver weights expressed as a percent of final body weights in male Wistar rats starved for one to three days were 2.4%, 2.2% and 2.1% respectively (67). The liver weight expressed as a percent of final body weight for the 45% feed restricted animals was a constant 2.8%. Therefore, we could foreseeably predict the liver weight by determining the level and duration of feed restriction or by estimating from the body weight. For example, if a male weanling Sprague Dawley rat was feed restricted at 45% for 4 weeks, the liver weight should be approximately 2.8% of the final body weight. However, one must be cautioned when making comparisons between liver sizes of ad libitum, starved and feed restricted animals in relation to liver function because of possible compositional differences even though the size may be the same. For example, in the ad libitum rats, the glycogen content would be expected to be much higher than that in starved or feed restricted rats.

The 100,000 x g supernatant protein content (mg/g liver) was not signficantly affected by the feed restriction (Table 11, page 78). However, the total 100,000 x g supernatant was significantly

decreased as a result of the feed restriction (Figure 13, page 80) and this again is more a reflection on the decrease in total liver size.

2. IN VITRO DRUG METABOLISM PARAMETERS

Hepatic microsomal proteins

The hepatic microsomal protein values when expressed per gram of wet liver tissue (Table 8, page 72) did not follow any consistent pattern at lower levels and shorter duration of feed restriction. This is indicative that at the lower levels and duration of the feed restriction, the animal's adaptive mechanism may be attempting to counter the effects of the restriction. This high variability of the protein values without a consistent pattern, prevented the possiblity of making any statistically significant inferences on the increase among the restricted groups. The feed restricted animals as a group had a significantly higher hepatic microsmal protein than the ad libitum controls (Figure 11, page 73). The hepatic microsomal protein of the animals feed restricted at 45% for four weeks was approximately 9.5% higher than that of the ad libitum animals (17.09 $\pm$ 0.90 vs 15.61 $\pm$ 0.07 mg/g liver). This higher hepatic microsomal protein (34%) in animals feed restricted at 45% for four weeks when compared to that in the ad libitum controls was also reported by Sachan (14) (8.57 $\pm$ 0.36 vs 6.39 $\pm$ 0.58 mg/g liver). Although the percentage increase in the current study was much lower than that reported by Sachan (14), the net amount of increase (1.49 and 2.18 mg) expressed as per gram of wet liver tissue were similar. The

values for the hepatic microsomal protein in the current study were higher than those reported by Sachan (14) but lower than those reported (27.0 $\pm$ 0.63 and 16.6 $\pm$ 0.58 mg/g liver for native microsomes and trypsin-digested microsomes respectively) by Marselos and Laitinin (8) and (23.3 $\pm$ 0.57 mg/g liver) by Kato and Gillette (10). One possible explanation for these wide margin of normal hepatic microsomal protein could be that the ad libitum animals under investigation were of growing age and thus have a rather rapid turnover of proteins resulting in the rather wide margin of normal hepatic microsomal protein values observed. In addition, the inducing agents in the feed consumed, the type of bedding utilized, and the use of pesticides or chemicals around the animals' housing environment could contribute to the observed variation of the hepatic microsomal protein values in ad libitum fed animals. Starvation for 72 hr, on the other hand, was reported by Marselos and Laitinin (8) to decrease the hepatic microsomal protein expressed as per gram of wet liver tissue while others reported no net effect (10,66-68). The reported decrease and no change in the hepatic microsomal protein content (mg/g liver) during starvation may be due to the catabolism of the protein for energy during starvation. Therefore, even though the liver weight was decreased, the amount of protein per gram of liver remained the same or decreased instead of increased as one might expect if no catabolism of the protein occured. Thus, feed restriction appears to result in synthesis of hepatic microsomal protein which is quite different from that observed during starvation. The highest amount of microsomal protein (mg/g liver) was

observed at the 45% feed restriction for five weeks. However, when the hepatic microsomal protein was expressed as the total content in the liver, feed restriction resulted in a decrease in the total hepatic microsomal protein content because of the smaller liver sizes in the feed restricted animals. The most severely feed restricted animals had the least total hepatic microsomal protein content (Table 9, page 74).

Hepatic cytochrome P-450

The pharmacologic action of a drug or environmental xenobiotic is dependent on it's metabolism by the drug metabolizing enzymes. Since cytochrome P-450 plays a key role in the Phase I of drug metabolism, the tissue content is often indicative of the drug metabolizing capacity. The significant increases in hepatic cytochrome P-450 content when expressed as per gram of wet liver tissue during feed restriction (Figure 14, page 82) was in accord with increases in the hepatic cytochrome P-450 after a 45% feed restriction for four weeks reported in the literature (14, 16). The 38% increase reported (0.65 $\pm$ 0.05 vs 0.47 $\pm$ 0.03 nmoles/mg protein) by Sachan (14) is comparable to the 30% increase (17.4 $\pm$ 0.82 vs 13.4 $\pm$ 0.43 nmoles/g liver) observed in the current study (Table 13, page 81). The increase in cytochrome P-450 along with the increase in the hepatic microsomal protein is suggestive of net cytochrome P-450 synthesis. This argument has been supported in earlier studies from our laboratory where hepatic DNA concentration were also found to increase (14). Starvation, on the other hand, has been reported to

have no effect on the cytochrome P-450 content (8, 66-69). The value of the cytochrome P-450 content of the ad libitum fed group in the current study when expressed as nmoles/mg protein (0.86 ± 0.01) is very similar to that reported (0.84 ± 0.06) by Nakajima and Sato (67). Although Nakajima and Sato (67) reported no significant differences among the values of the hepatic cytochrome P-450 content of the animals starved for 0, 24, 48 and 72 hr, closer examination of the data indicated that there was a trend of decreasing hepatic cytochrome P-450 content with increasing duration of starvation (0.842 ± 0.123, 0.823 ± 0.030, 0.782 ± 0.042 and 0.799 ± 0.090 nmoles/mg protein at 0, 24, 48 and 72 hr of starvation respectively). In contrast to starvation, increasing levels of feed restriction resulted in a significant linear increase in cytochrome P-450. This may be interpreted as an increase in Phase I drug metabolizing capacity provided that sufficient reducing equivalents and drug metabolizing enzymes were present. On the other hand, if the total cytochrome P-450 contents per liver are compared, feed restriction resulted in significant linear decreases in cytochrome P-450 (Figure 20). This ought not be surprising since the marked reduction in liver weight with the feed restriction is far greater than the small increase in the cytochrome P-450 observed as per gram of wet liver tissue.

Aniline Hydroxylase

In order to further examine the effects of feed restriction on drug metabolizing systems, model substrate compounds were employed

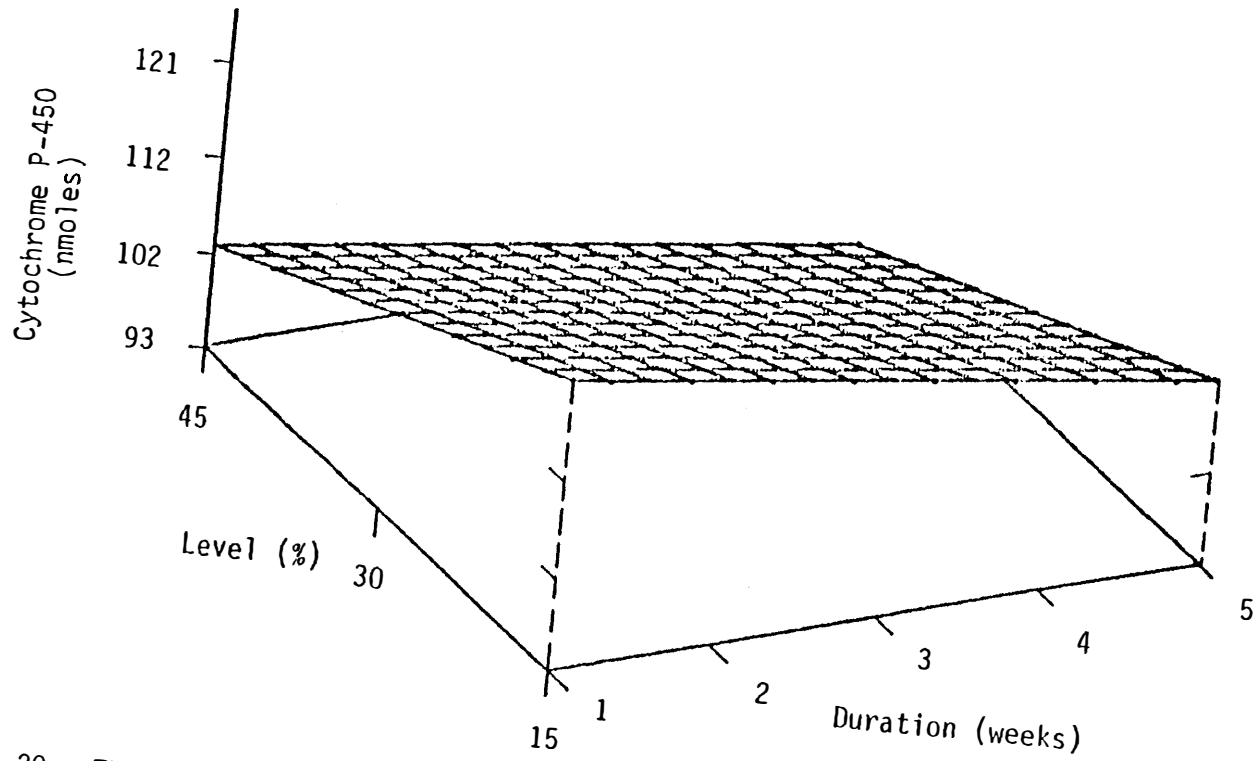


Figure 20. The effect of different levels (L) and duration (D) of feed restriction on the total hepatic cytochrome P-450 content (TP-450).
TP-450 = 132.47 - 2.55 D - 0.60 L ($P \leq 0.05$).

to assess in vitro activities of drug metabolizing enzymes. The hepatic aniline hydroxylase activity was significantly increased as a result of feed restriction (Figure 16, page 88). The linear increase was significant with the increasing levels and duration of feed restriction. The 45% feed restricted animals for four weeks had a 47% higher in vitro activity of the enzyme than the ad libitum group (14.8 $\pm$ 0.89 vs 10.1 $\pm$ 0.47 nmoles HCHO formed/min/g liver). This is akin to the 42% higher in vitro activity reported (12.25 $\pm$ 0.85 vs 8.60 $\pm$ 1.41 nmoles HCHO formed/min/g liver) by Sachan (14). Aniline hydroxylase is one enzyme that is shown to increase (64%) by starvation (10). The activity of this enzyme for the ad libitum fed rats is similar to that reported by Kato and Gillette (10) (237 $\pm$ 7.16 nmoles/30 min/g liver) which lends validity to our determinations. The total in vitro aniline hydroxylase activity was decreased by feed restriction and both degree and duration of restriction exerted significant influence. The 45% feed restriction for four weeks lowered total in vitro aniline hydroxylase activity approximately 25% in the current study which was similar to the 36% lower total in vitro activity observed in the 45% restricted animals by Sachan (14).

PCMA-N-demethylase

The activity of PCMA-N-demethylase was unaffected by the feed restriction (Table 17, page 87). The total in vitro activity on the other hand was significantly decreased with increasing levels of feed restriction (Figure 21). Sachan (14) reported a 73% higher (13.07 $\pm$

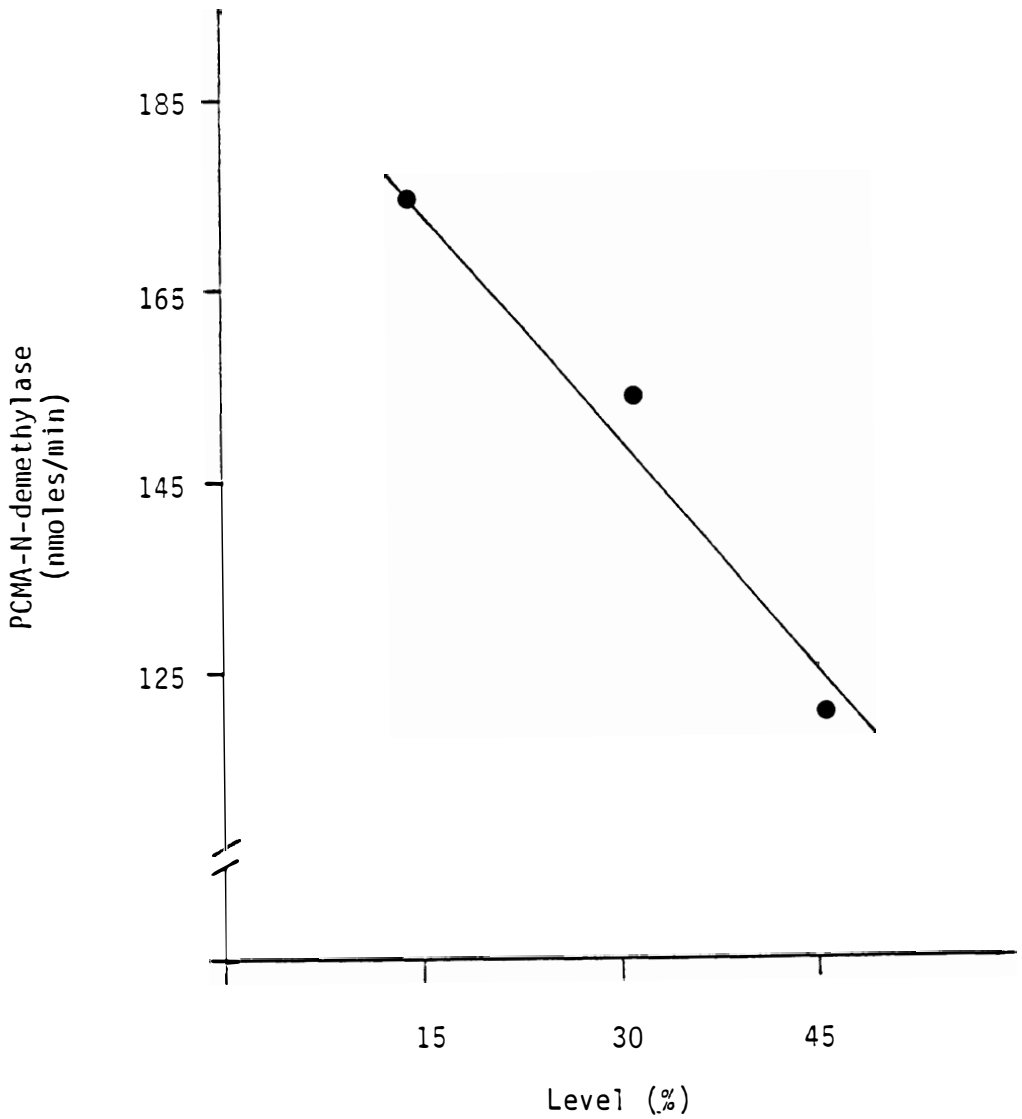


Figure 21. The effect of different levels (L) of feed restriction on the total in vitro activity of hepatic PCMA-N-demethylase (TPCMAD). TPCMAD = 196.70 - 1.58 L ($P \leq 0.05$). ● n = 25.

1.55 vs 7.54 ± 2.78 nmoles PCA formed/min/g liver) in vitro activity of hepatic PCMA-N-demethylase when the animals were feed restricted at 45% for four weeks. However when this activity was expressed as the total activity, a 22% decrease was observed.

UDP-glucuronyl transferase

The p-nitrophenol-UDP-glucuronyl transferase activity was also unaffected by the feed restriction (Table 18, page 90). Sachan and Das (16) reported a significant increase in the enzyme activity in rats feed restricted at 50% for 49 days (0.693 ± 0.02 vs 0.578 ± 0.02 μmoles of product formed/hr/g liver). The total enzyme activity however, was decreased just as in the present study (Figure 22). In their study, the substrate used was 4-methyl umbelliferone, whereas, the substrate used in the current study was p-nitrophenol. Discrepancies in the measured enzyme activity from using different substrates have been reported by Duvaldestin et al (71).

Cytochrome c reductase

The in vitro activity of hepatic cytochrome c reductase was significantly decreased with feed restriction, regardless of whether it was expressed as per gram of wet liver tissue or as total activity (Figures 15, page 85 and Figure 23). This decrease in total cytochrome c reductase activity was significantly linear with increasing levels of feed restriction. The effects of feed restriction on the activity of this enzyme is not well documented.

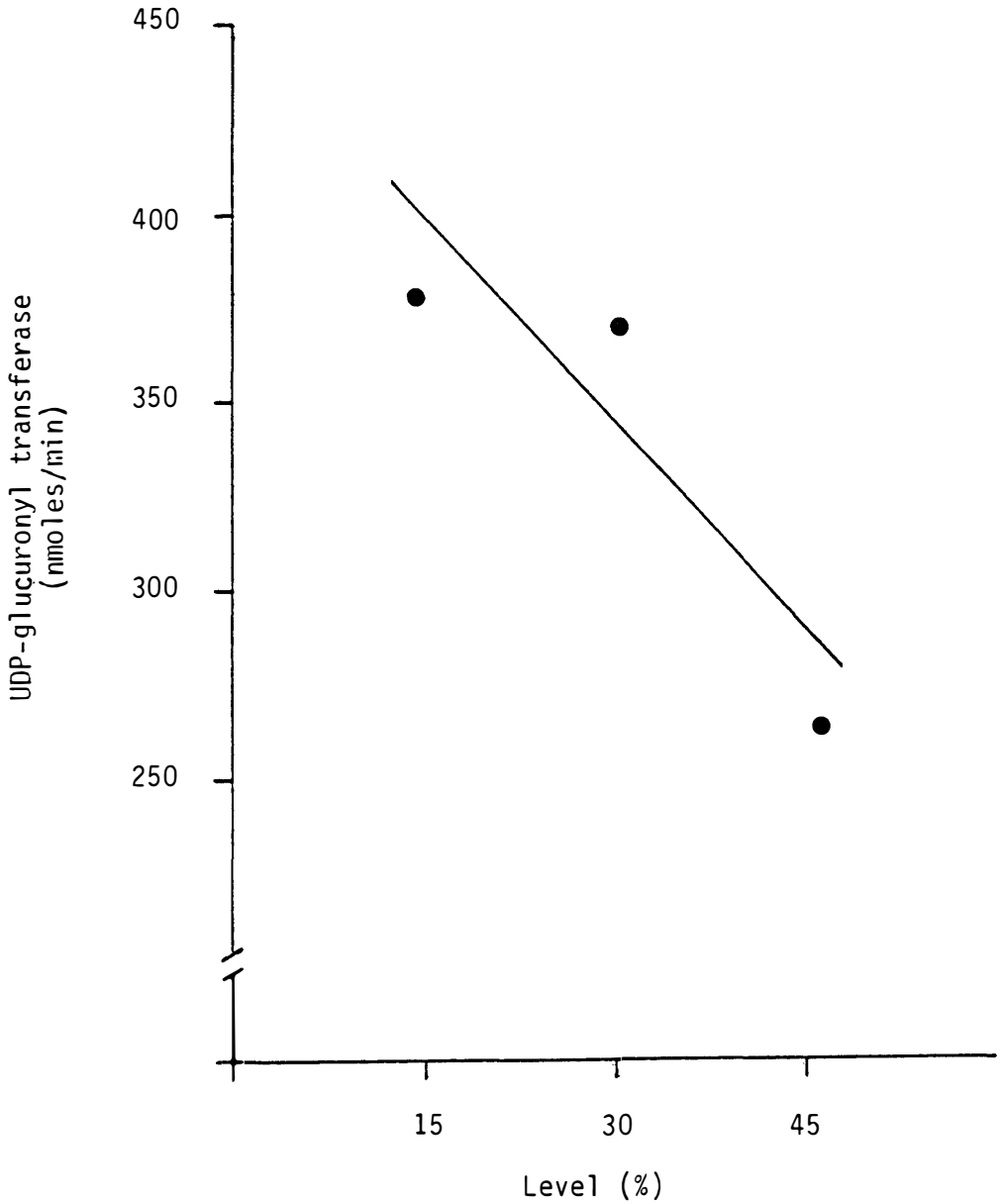


Figure 22. The effect of different levels (L) of feed restriction on the total in vitro activity of hepatic UDP-glucuronyl transferase (TGT). TGT = 457.16 - 3.71 L ($P \leq 0.05$).
● n = 25.

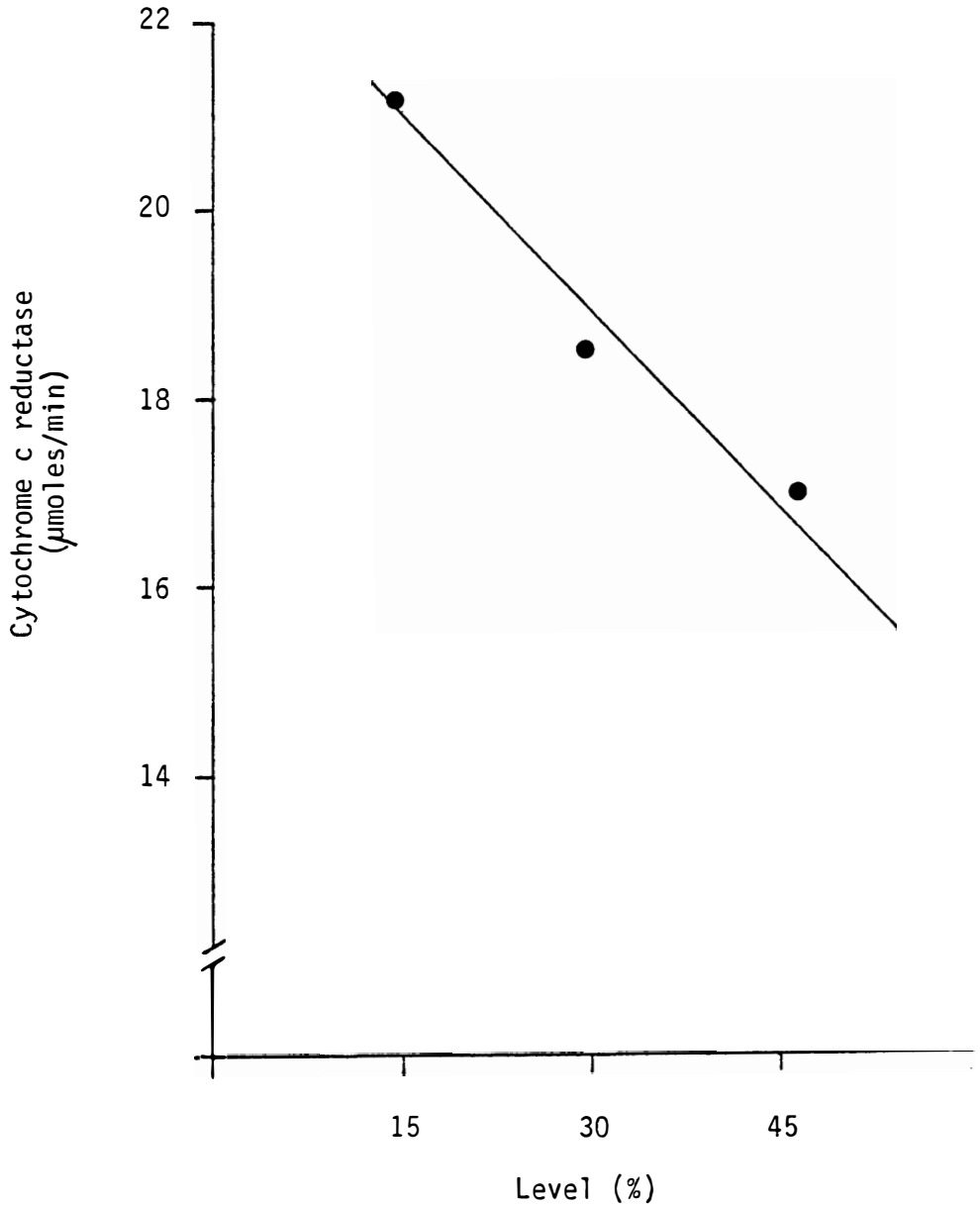


Figure 23. The effect of different levels (L) of feed restriction on the total activity of hepatic cytochrome c reductase (TCYTC). TCYTC = 23.225 - 0.144 L ($P \leq 0.05$).
● n = 25.

However, earlier studies (unpublished results) in our laboratory indicated that the in vitro cytochrome c reductase activity (nmoles cytochrome c reduced/min/g liver) was lower (21%) in the animals restricted at 15% and 30% for four weeks when compared to the ad libitum animals. The value of the cytochrome c reductase activity of the ad libitum group observed in the current study was slightly higher (3.56 $\pm$ 0.13 μmoles cytochrome c reduced/min/g liver vs 84.60 $\pm$ 2.68 μmoles cytochrome c reduced/30 min/g liver) than that reported by Kato and Gillette (10). This discrepancy in the values may be due to the lower hepatic microsomal protein values (mg/g liver) observed in the current study when compared to that of Kato and Gillette (10). If the lower hepatic microsomal protein value was due to experimental error, the under estimation of the microsomal protein content could foreseeably result in an over estimation of the cytochrome c reductase value. However, this is not very likely since the cytochrome P-450 values and other enzyme values were quite comparable with those reported in the literature. In the current study, the cytochrome c reductase activity was assayed within 24 hours of sacrifice whereas it is not known how soon the activity was measured in the study by Kato and Gillete (10). Since more than 15 different enzymes were assayed in their study, it is possible that cytochrome c reductase activity was not assayed in fresh preparations which may bave resulted in a decrease of the enzyme activity due to storage.

NADPH-generating enzymes

Since NADPH is an essential cofactor in the metabolism of drugs, alterations in the NADPH-generating enzymes could provide further insight to the drug metabolism. The activities of hepatic NADPH-generating enzymes, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and malic enzyme were all significantly enhanced by the feed restriction when expressed as per gram of wet liver tissue (Figures 17, 18, and 19, pages 94, 96, and 98 respectively). These findings are consistent with those found in the literature where 50% feed restriction for 49 days resulted in increases of 82% (14.09 $\pm$ 1.40 vs 7.76 $\pm$ 1.04 μmoles of substrate utilized/min/mg protein) in malic enzyme, 97% (22.96 $\pm$ 2.87 vs 11.67 $\pm$ 2.40 nmoles substrate utilized/min/mg protein) in glucose-6-phosphate dehydrogenase, and 98% (33.88 $\pm$ 1.93 vs 17.06 $\pm$ 1.90 nmoles substrate utilized/min/mg protein) in 6-phosphogluconate dehydrogenase enzyme activities. The ad libitum values of malic enzyme, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase (10.33 $\pm$ 0.86, 12.51 $\pm$ 1.40, and 45.09 $\pm$ 2.03 nmoles NADPH formed/min/mg protein, respectively) in the current study were very similar to that reported by Sachan and Das (16). Starvation, on the other hand, resulted in decreases in all three enzymes when expressed as per 100 g of body weight (9).

When expressed as the total activity in the liver, 6-phosphogluconate dehydrogenase a significant linear decrease was observed with increasing levels and duration of feed restriction (Figure 24) while glucose-6-phosphate dehydrogenase increased

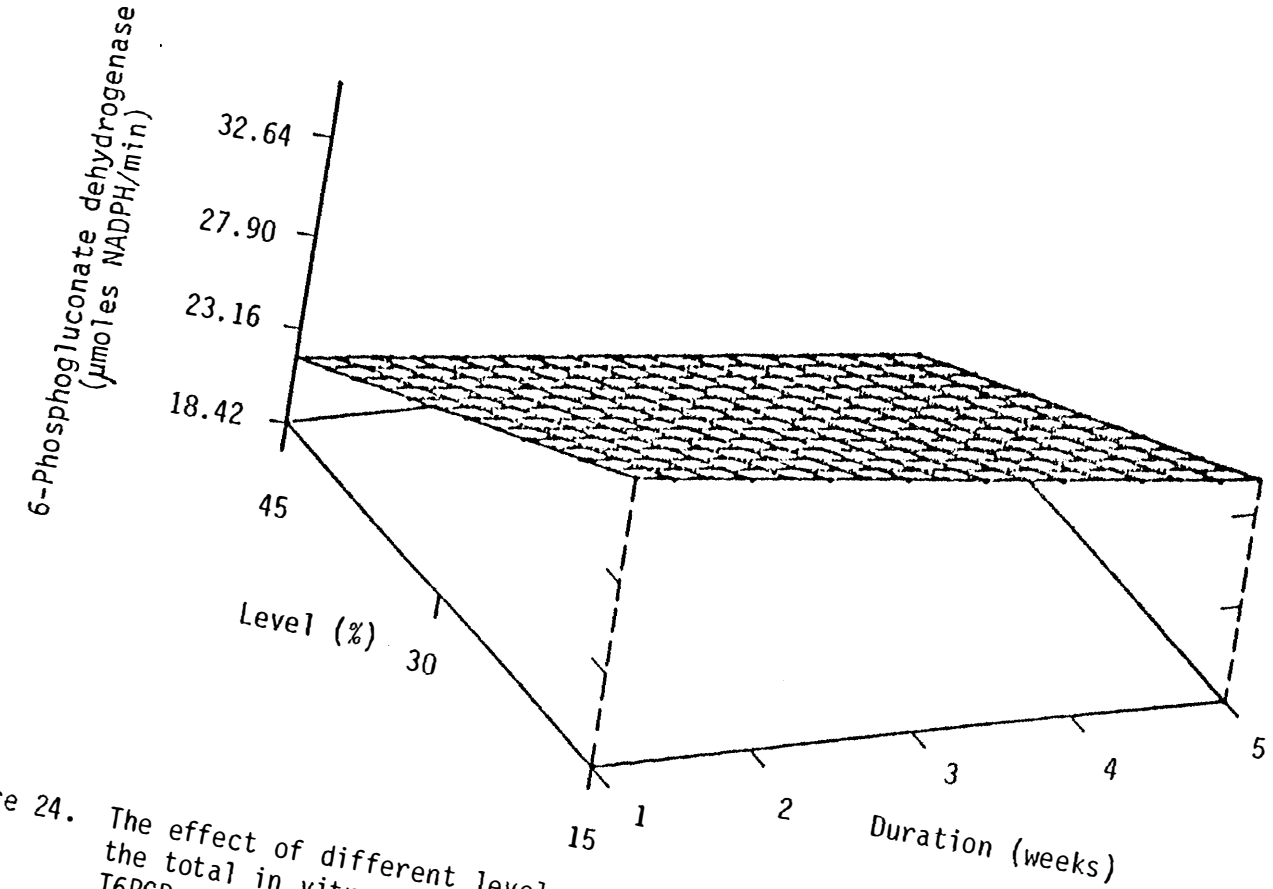


Figure 24. The effect of different levels (L) and duration (D) of feed restriction on the total in vitro activity of hepatic 6-phosphogluconate dehydrogenase (T6PGD). T6PGD = 38.92 - 0.827 D - 0.364 L ($P \leq 0.05$).

linearly with the increasing duration of feed restriction (Figure 25). Malic enzyme, however,increased linearly with the increasing duration but decreased curvilinearly with the increasing levels of feed restriction (Figure 26).

Thus, the increasing levels and duration of feed restriction results in increased in vitro activity of the aniline hydroxylase which is in concert with increased cytochrome P-450 content and microsomal protein. PCMA-N-Demethylase and p-nitrophenol UDP-glucuronyl transferase activities remained unchanged while cytochrome c reductase activity was decreased by the feed restriction. The inconsistencies in the changes in the vitro activities of these drug metabolizing enzymes during feed restriction were not unexpected in view of the findings in the literature (14,16). Not all enzymes change in concert with the changes in cytochrome P-450 and it must be pointed out that one cannot generalize about the activities of the enzymes based on a one substrate model. For example in the study of the enzyme UDP-glucuronyl transferase using three different substrates bilirubin, phenolpthalein and p-nitrophenol, Duvaldestin et al (71) reported that only the bilirubin UDP-glucuronyl transferase activity was increased in the rats starved for three days when compared to the enzyme activity in the controls. Glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and malic enzyme were all significantly increased with increase in severity of the feed restriction.

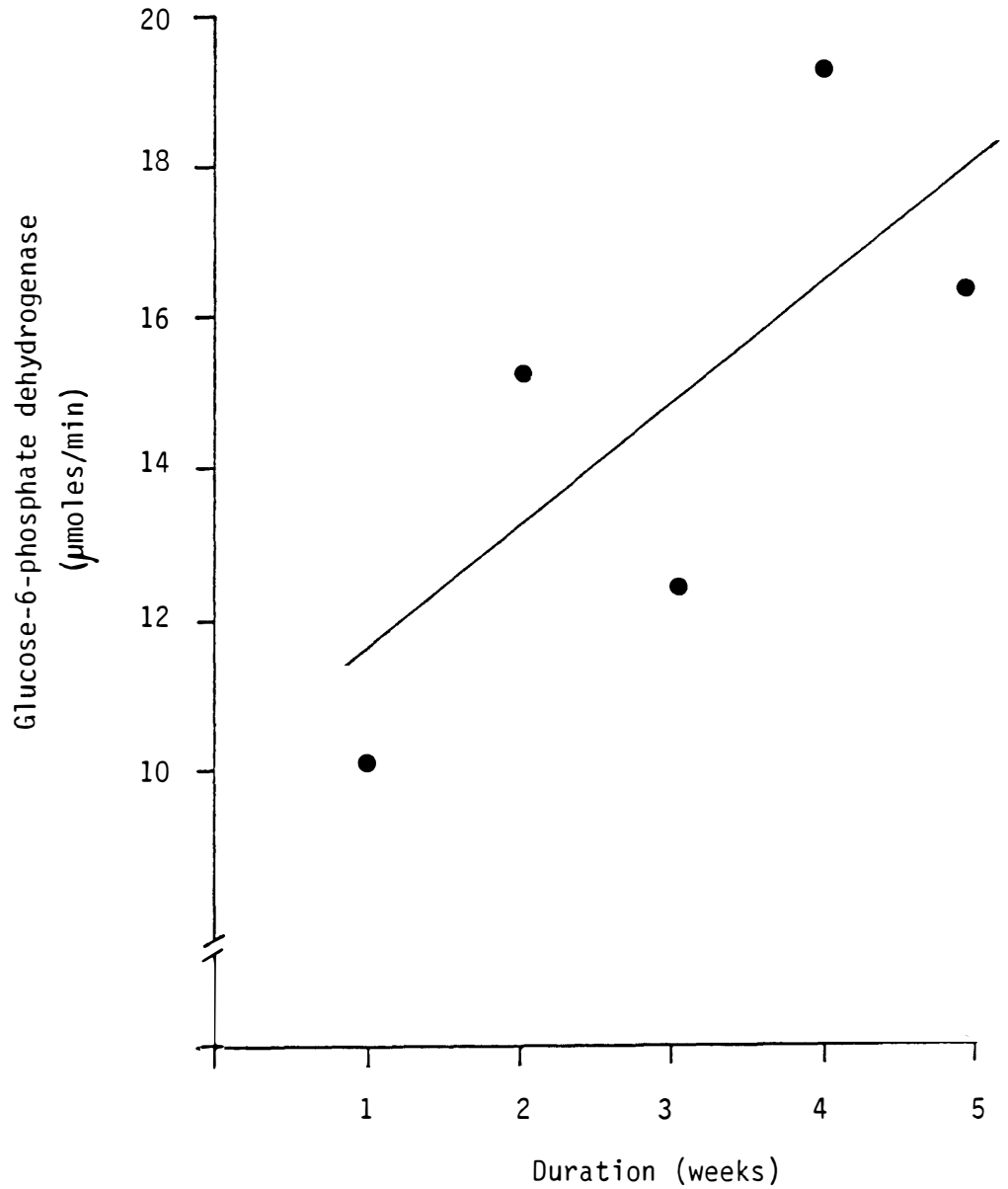


Figure 25. The effect of duration (D) of feed restriction on the total in vitro activity of glucose-6-phosphate dehydrogenase (TG6PD). TG6P = 10.14 + 1.60 D ($P \leq 0.05$). ● n = 15.

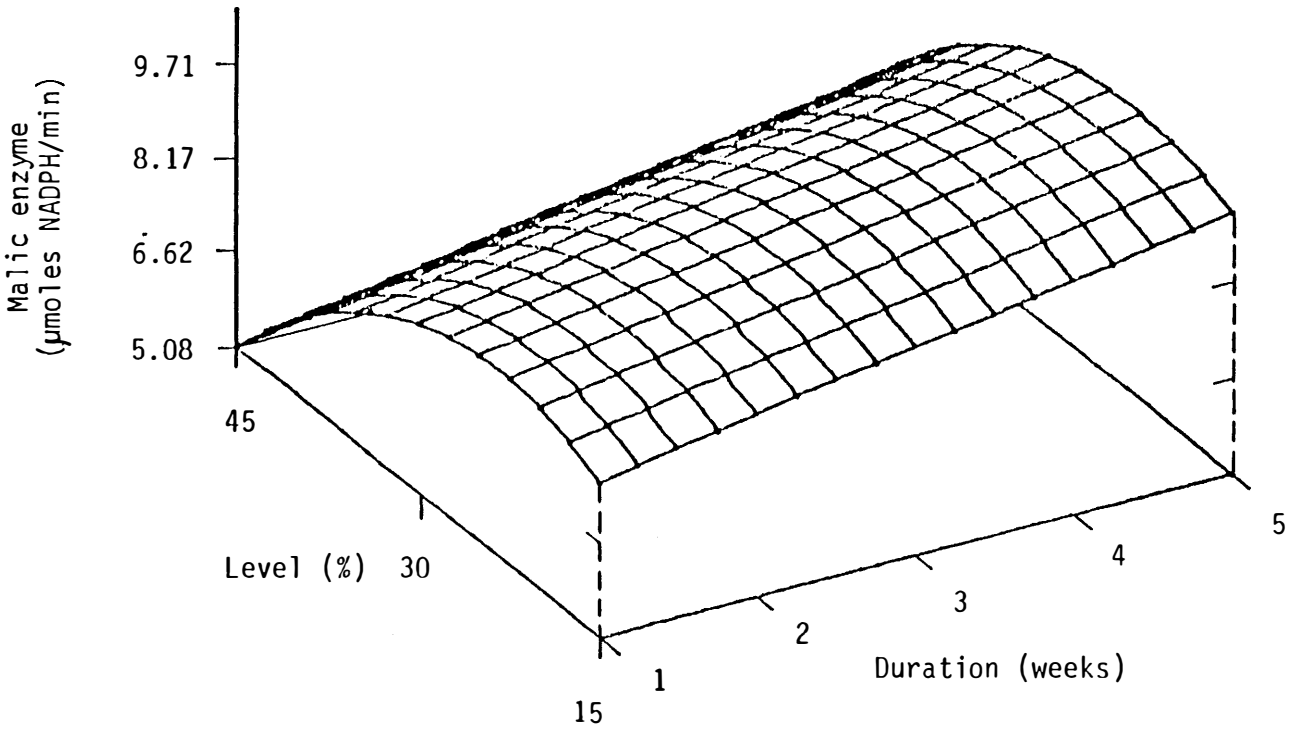


Figure 26. The effect of different levels (L) and duration (D) of feed restriction on the total in vitro activity of hepatic malic enzyme (TME).

$TME = 4.051 + 0.413\ D + 0.306\ L - 0.006\ L^2$ $(P \leq 0.05)$.

These observations (expressed as per gram of wet liver tissue) are quite unlike that noted during starvation or deficiencies of single nutrients in the diet. During starvation, cytochrome P-450 was unchanged (8,66-69), UDP-glucuronyl transferase was increased (8,71), cytochrome c reductase was decreased (10) and the NADPH generating enzymes (glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and malic enzyme) were decreased (9). Aniline hydroxylase however was increased during starvation (10). During the deficiencies of single nutrients, protein (35-38), dietary lipid (48) and specific vitamins (53,58-64) decreases in the activities of hepatic drug metabolizing enzymes were observed. Carbohydrate deficient diets were also reported to result in decreased hepatic cytochrome P-450 content, and aminopyrine demethylase activity but increased p-nitrophenol UDP-glucuronyl transferase activity (3).

It has been suggested that the drug metabolizing capacity may in part be dependent on the availability of NADPH (65,93). Presumably the restricted diets in the current study even at 45% restricted for 5 weeks were not really deficient in any of the nutrients. Usually, enhanced activities of NADPH-generating enzymes are associated with lipogenesis. However, at the higher levels and/or longer duration of feed restriction, the animal's anabolic system would not be actively engaged in lipogenesis. Thus the higher activities of NADPH-generating enzymes observed during feed restriction could possibly contribute higher levels of NADPH to enhance the pathway for the metabolism of drugs and foreign compounds. Coupled with this,

the animal's catabolic system may be engaged in lipolysis (especially during more severe feed restriction). This could result in the release of lipophilic drugs or foreign compounds that have been accumulated in the adipose tissues and in turn induce the drug metabolizing enzymes over a period of time. However, it must be pointed out that the feed restricted animals have a smaller liver and this in most instances translates to lesser total amount of the liver enzyme per animal. This is especially relevant in the case of an accidental dose of a drug where all animals regardless of size are exposed to the same dose. In this case, the feed restricted (smaller) animal with a lesser total amount of drug metabolizing enzymes would presumably be more susceptible to the pharmacologic effects. Therapeutic doses based on per unit body weight, on the other hand, could introduce yet another interpretation of the implications of this study. From this phase of the study, it can be concluded that increasing severity of feed restriction in healthy weanling rats results in a higher drug metabolizing capacity per gram of wet liver tissue and the level or duration of feed restriction required to affect this higher drug metabolism rate depends on the level of increase sought. Each enzyme appears to respond to the different levels and duration of feed restriction in an individual manner. When the enzyme activities are expressed as per whole liver, feed restriction resulted in a decrease in the total activities.

2. IN VIVO DRUG METABOLISM DURING 45% FEED RESTRICTION

The following two experiments were performed to substantiate the restriction-induced changes in the in vitro hepatic drug metabolizing enzymes and the in vivo metabolism of a drug. The results from Experiment I indicated that a 45% feed restriction for four weeks was sufficient to cause maximum changes in the hydroxylating enzymes. Antipyrine has been extensively used for the study of microsomal drug metabolism (94) and its pharmacokinetics are widely used as an indicator of the rate of in vivo drug metabolism in man (95-97) and animals (98,99). Antipyrine is metabolized extensively by the hepatic microsomal enzyme (100) and less than 10% of the parent compound is bound to plasma proteins (101). Therefore, it was chosen to be the model drug for this study.

The volume of distribution and blood half life of antipyrine in the current study were slightly lower (145 $\pm$ 15 vs 192 $\pm$ 41 ml and 53 $\pm$ 2 vs 87 $\pm$ 4 min, respectively) than the values reported by Knights et al (99). The blood clearance on the other hand was slightly higher (1.92 $\pm$ 0.24 vs 1.53 $\pm$ 0.33 ml/min). The results in Experiment II demonstrated that in feed restricted animals antipyrine had a shorter blood half life than in the ad libitum animals (47.22 $\pm$ 1.91 vs 53.00 $\pm$ 1.75 minutes). This is complimentary to the increased in vitro activities of hepatic drug metabolizing enzymes of the feed restricted animals. Increased hepatic drug metabolizing enzyme activities and decreased hexobarbital sleeping time observed by Sachan (14) also lend support to this data. The distribution volume of antipyrine in the restricted animals was also significantly lower

than that in the ad libitum animals which may be attributed to the smaller size of the restricted animals. Since blood clearance is also significantly lower in the restricted animals which can be related to the volume of distribution and consequently the weight or size of the animal. While the results of the current study are in agreement with the human data reported by Krishnaswamy and Naidu (95), these observations differ from those of Narang et al (96) and Tranvouez et al (97). In the study by Krishnaswamy and Naidu (95), the antipyrine half life in undernourished subjects was shorter than that in the normal non-smokers. However, the undernourished subjects with nutritional edema had prolonged antipyrine half lives suggesting an impairment of hepatic function due to liver damage. The retention of fluids in the subjects with edema may also increase the volume of distribution for the drug and thus reduce its clearance. Tranvouez et al (97) reported that subjects with energy malnutrition had significantly decreased metabolic clearance rate and volume of distribution but not the antipyrine half life when compared to the control subjects. The subjects with protein-calorie malnutrition, on the other hand, had decreased metabolic clearance rate and volume of distribution but increased antipyrine half life again indicating impairment of hepatic function. These results are consistent with findings in the literature of increased hexobarbital metabolism during feed restriction (14). Thus it appears that unless there is an impairment of liver function, reduced food intake does not result in increase in the blood half life of antipyrine and could result in an increase metabolism of the drug in rats.

Experiment III was conducted to determine if the increased in drug metabolizing enzyme activities observed during feed restriction actually translated to an increase in toxicity of a drug that is 'activited' by the mixed function oxidase system. It is known that carbon tetrachloride is metabolized by the hepatic microsomal drug-metabolizing enzymes to reactive intermediates that destroy membrane structures and caused lipid oxidation (102-105). The LD_{50} dose of 5.4 mg/kg body weight resulted in a mortality rate of 70% in the ad libitum animals and 80% in the 45% feed restricted animals. The three surviving animals in the ad libitum group were not showing any morbidity from the dose at the end of a week post dosage while the one surviving animal in the 45% feed restricted group was still off feed and had nasal exudates and a rough hair coat. The majority of the mortality of animals in the 45% feed restricted group occurred early during the week after carbon tetrachloride administration while the mortality of the ad libitum group was more spread out over a five day period post carbon tetrachloride dosage. The liver weight as a percentage of the body weight of the restricted animals was significantly higher than that of the ad libitum animals indicating perhaps a greater amount of fatty deposits in the liver. However, due to the small number of animals, the data should only serve as a basis for future experimentation.

Perhaps a more appropriate approach of investigating the in vivo toxicty of a compound under the conditions of feed restriction would have been to determine the LD_{50} dose of the compound under the prescribed conditions. For example, 40 animals would have been

randomly divided into eight subgroups of five animals each. This eight subgroups would then have been randomly assigned to the four different dose levels in the ad libitum and feed restricted groups. At the end of the feeding period, the animals in each subgroup would have been given suitable doses of the carbon tetrachloride and monitored for the mortality at each dose level. The LD_{50} value would then have been calculated using the moving average method (106).A pilot study may have been necessary to determine the approximately range of dosage to be used in the feed restricted animals since no LD_{50} data available.

The five animals per dosage and four dosage levels proposed here are by no means absolute since tables have been constructed for the use of as few as two animals per dosage with four or more dosage levels being tested (107). In toxicity testing, many regulatory standards and guidelines (for example by the Interagency Regulatory Liaison Group) require the use of at least five animals per sex per dosage level and at least three dosage levels (106). Although LD_{50} determination imparts valuable information, ethical and humane considerations should always be made to weigh any possible suffering of the animals against the value or need of the the generated data.

The overall conclusion of these experiments is that feed restriction results in changes of hepatic microsomal enzyme activities that are quite different from starvation or deficiency of single nutrients. The interpretation of the result and any implications derived should take into account the way the enzyme activity is expressed. Pharmacological studies and/or therapeutic

modalities should take into account the nutritional state of the animals.

Significance of feed restriction and drug metabolism

We are constantly exposed to increased amounts of xenobiotics such as drugs, toxins, pollutants, pesticides, food additives, food colors, potential mutagenic and carcinogenic agents (108). The ability and the manner of our body's capacity to metabolize these compounds would determine our very survival and adaptation to our environment. For example, the efficacy, toxicity and safety of a wide range of drugs are determined by our drug metabolizing capacity. Many carcinogens also require 'activation' by the mixed function oxidase system to exert their carcinogenic effect. Also knowledge of the effects of partial food restriction on the drug metabolizing system would enhance, if not be necessary for optimum therapeutic response of drugs, especially those with narrow therapeutic margin and individualization of drug therapy. The animals used in the current study were growing animals and the extrapolation of the results to humans would thus be more applicable to children. The age at the onset of restriction was different for each treatment group and this cannot be compensated easily although an alternative would be to use adult animals that have reached a constant weight. Extrapolation of the results should be viewed with caution since drug metabolism is known to be subjected to a great deal of variation between species as well as in the same species (30). Some people have dramatic changes in their drug metabolism capacity in response to

dietary changes while others do not (108). These great variablity or individuality of response in human studies could be the result of genetics, sex and environment (7).

Although the result in this study demonstrated that drug metabolizm was enhanced during partial food restriction in rats, the extrapolation of these results to humans especially in the case of malnutrition is complicated by a number of factors: Nutritional deficiencies as well as fatty infiltration of the liver usually accompany severe protein-calorie malnutrition. Decreases in absorption, transit time and body fluids as well as accompanying disease states could also result in a different drug metabolizing capacity (108). Concurrent medication as well as prior exposure to other inducers of the mixed function oxidase system (cigarette smoke for example) could alter the inducibility of the very system itself (27). A vegetarian diet containing a relatively high amount of inducers could foreseeably result in enhanced xenobiotic metabolism (2) which may take a different course upon food restriction. Since feed restriction enhances the mixed function oxidase system, it is possible that the metabolism of certain endogenous substrates may also be modified and therefore, alter their effective levels in the body. Thus the increases in drug metabolism observed in the feed restricted male weanling rats may be due to decreased androgen levels, leading to a status like those of female rats where the drug metabolizing enzyme activities were enhanced by starvation due to the lack of androgens.

CHAPTER VI

SUMMARY

The effects of increasing levels and duration of feed restriction on drug metabolism was examined in an animal model. Increasing levels and duration of feed restriction from 15% for one week to 45% for five weeks resulted in significant progressive decreases in the amount of body weight gain and the liver weight. The decrease in body weight gain with the increasing levels and duration of feed restriction was curvilinear in both directions. The least amount of weight gain was observed in animals restricted at 45% for 5 weeks. The liver weight changes also followed similar patterns. These decreases were in direct response to the reduced feed consumption.

The hepatic microsomal protein content was increased in the feed restricted animals. Although the level of microsomal protein content in the animals restricted at the lower levels and shorter duration varied, a more consistent pattern was observed at the higher levels of restriction. The soluble proteins (100,000 x g supernatant proteins) were unaltered by the feed restriction.

Cytochrome P-450 content was significantly increased in the animals subjected to the increasing levels of feed restriction. This increase was linear with the level of feed restriction only. The highest amount of cytochrome P-450 content was observed in the animals feed restricted at 45% for four weeks. The in vitro activilty of the associated enzyme, NADPH-cytochrome c reductase, was decreased in the restricted animals although no consistent pattern for this

decrease was apparent. The in vitro hepatic aniline hydroxylase activity of the restricted animals increased linearly with both the level and duration of feed restriction while PCMA-N-demethylase and p-nitrophenol UDP-glucuronyl transferase activities were unaltered by the feed restriction. The in vitro activities of the NADPH-generating enzymes, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and malic enzyme were also enhanced by feed restriction. Glucose-6-phosphate dehydrogenase activity increased linearly with the duration of feed restriction only while 6-phosphategluconate dehydrogenase activity changed linearly with the duration of feed restriction but curvilinearly with the level of feed restriction. Malic enzyme activity increased linearly with the duration of feed restriction and curvilinearly with the level of feed restriction. However, when the total amount of these drug metabolizing parameters were assessed, a decrease was apparent.

The primary changes observed here are unlike those in animals during starvation or single nutrient deficiences. The activity of drug metabolizing enzymes including cytochrome P-450 is inversely related to the plasma half life of a drug in the animals. It was shown by the data of Experiment II, that 45% feed restricted animals metabolized antipyrine significantly faster (shorter $T_{1/2}$) than the ad libitum controls. The carbon tetrachloride study, although not conclusive, supported this point of view. Thus it is concluded that increasing severity of feed restriction resulted in enhanced drug metabolism and that a 45% feed restriction was most effective in bringing about the increase in drug metabolizing parameters.

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