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# DIETARY EFFECTS ON MIXED FUNCTION OXIDASE ACTIVITY IN THE RUMINANT

A Thesis

Presented for the

Master of Science

Degree

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Kenneth P. Zanzalari

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# ABSTRACT

The objectives of this research were to determine effects of age, heat-browned hay and protein intake on MFO activity in ruminants. A preliminary study determined that rats fed browned egg albumin had higher hepatic cytochrome P-450 concentrations than rats fed a similar diet, excluding the browned egg albumin. Holstein steer calves were then utilized in three experiments to determine the effects of age, heat-browned hay and protein level. MFO activity was assessed by measuring the rate of metabolism (disappearnce from blood plasma) of antipyrine, a compound extensively metabolized in the liver (Anderson et al., 1982). Calves were dosed with 18 mg/kg B.W. antipyrine via the jugular vein and blood samples were taken from the opposite vein at 0, 0.5, 1, 2, 3, 4 and 5 hr post dosing.

Results indicated that antipyrine was metabolized faster at 1 mo of age than at either 2, 3 or 4 mo. Disappearance rates of antipyrine were 72.7, 45.8 and 60.7% faster ($P < .05$) at 1 mo than at 2, 3 or 4 mo, respectively. Heat-browned hay and protein content of the diet had no effect on MFO activity. In an experiment conducted on catherterized sheep in which blood flows and liver extraction ratios could be measured antipyrine clearance by the liver accounted for 10 to 20% of the initial dose. It was concluded that the remaining 80 to 90% was cleared via the saliva which carried antipyrine to the rumen where it was finally metabolized.

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# CHAPTER I

## INTRODUCTION

The efficient utilization of energy by animals for production is a major concern for the producer. Feed costs are the single highest expense on farm operations. Therefore, the efficient utilization of energy could cut costs significantly while maintaining or increasing production. The need for more efficient production is evident due to increasing world population in association with higher costs for production and decreasing farm numbers. A greater production need will eventually arise and a possible solution may be increased efficiency of metabolic regulation. Many metabolic systems have been identified as energy wasteful. One of the more wasteful is the mixed function oxidase system, referred to in the literature as MFO or P-450. Since the MFO is energy wasteful, a decrease in it's activity could make an animal more energy efficient and allow it to gain more weight (Smith, 1982) or produce more milk at the same level of energy intake.

Many factors including diet composition, cooking method and various drugs have been shown to alter the activity of the MFO in humans and laboratory animals. However, little work has been conducted on ruminants and knowledge of these types of interactions could prove invaluable.

In ruminants, as well as nonruminants, the activity of the MFO determines the rate of metabolism, excretion and, ultimately, the residual time that chemicals or toxins remain within animal tissues

(Burley and Bray, 1983). Mixed function oxidases are essential for herbivores due to intake of the so-called secondary substances of plants, i.e. compounds such as phenols, quinones, terpenoids and alkyloids which are widely distributed in plants and frequently are repellent or toxic to animals (Brattsten and Wilkinson, 1977). However under present feeding practices exposure to these toxicants may be minimal.

These observations pose the question: can the MFO be manipulated to increase efficiency of feed energy utilization without decreasing the animals natural defense mechanism against toxic chemicals such as secondary substances of plants?

Therefore, the objectives of our studies were to determine the effects of age, Maillard reaction products and protein level on MFO activity in calves.

# CHAPTER II

# REVIEW OF LITERATURE

## MFO as an Energy Wasteful System

The MFO is considered to be one of several energy wasteful systems since oxidation of both substate and cofactor occur without the production of a high energy compound such as ATP (Pirola and Lieber, 1975). Smith (1981) considers the MFO to be the most energy wasteful on a per carbon atom basis. At the oxidation level of pyridine nucleotides the MFO utilizes four atoms of hydrogen resulting in a loss of six ATP potentials since two hydrogen atoms at the oxidation level may generate three ATP'S from ADP and inorganic phosphate (Smith, 1981). It has been demonstrated that induction of hepatic microsomal enzymes (cytochrome P-450-dependent system) can significantly alter the body's metabolic efficiency since oxygen consumption and weight gains have been observed to be negatively correlated in rats given known inducers of microsomal enzymes (Pirola and Lieber, 1975). Dorsey and Smith (1981) suggest that the reverse situation, i.e. reduced cytochrome P-450 levels and, consequently, decreased MFO activity could result in the conservation of metabolic energy.

Heat increment is an important component of the total energy expenditure of lactating dairy cows. Coppock et al. (1981) stated that "as milk production continues to increase, greater metabolic heat production will occur concomitant to metabolism of nutrients to support that high production." Coppock et al. (1981) also states that

"in areas with high ambient temperatures, shade management to reduce heat stress will be especially valuable." However, another possibility of maximizing efficiency would be minimizing stimulation of energy wasteful metabolic systems (i.e. MFO) through choice of feeds which do not induce excessive activity.

Role of MFO

Primary drug metabolism occurs in the liver through the MFO and several conjugation enzymes, including sulfotransferases (ST), UDP-glucuronyl transferases (GT), and glutathione-S-transferases (GSH-T). This system efficiently removes and metabolizes drugs and their metabolites (Bidlack et al., 1986). The MFO alters functional groups through N- or O- dealkylations or hydroxylations making the substrate more water soluble. The next group of enzymes specifically conjugates these sites with endogenous cofactors such as sulfate, glucuronic acid, and glutathione (Bidlack et al., 1986). MFO enzymes are attached to the endoplasmic reticulum of cells in association with an electron transport pathway. Coon (1978) determined that three fractions are required for MFO substrate hydroxylation. First, a soluble form of cytochrome P-450 (P-450) identified spectrally as the carbon monoxide complex, secondly, a soluble form of NADPH-cytochrome c reductase (P-450 reductase), and thirdly, a heat-stable, chloroform-soluble phospholipid of which phosphatidylcholine is the most active. Figure 1 illustrates the electron transfer necessary for substrate hydroxyation and Figure 2 the proposed mechanism af action.

Cytochrome P-450 exists in several forms, each isozyme having different properties such as spectral characteristics, molecular

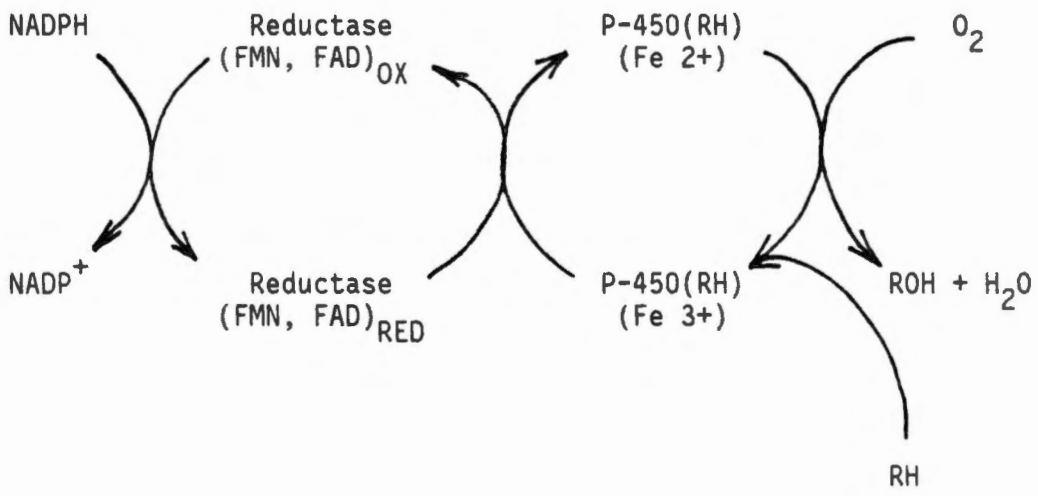


Figure 1. Scheme illustrating electron transfer reactions involved in substrate hydroxylation in the reconstituted enzyme system.

From Coon. 1978. Nutr. Rev. 36:322.

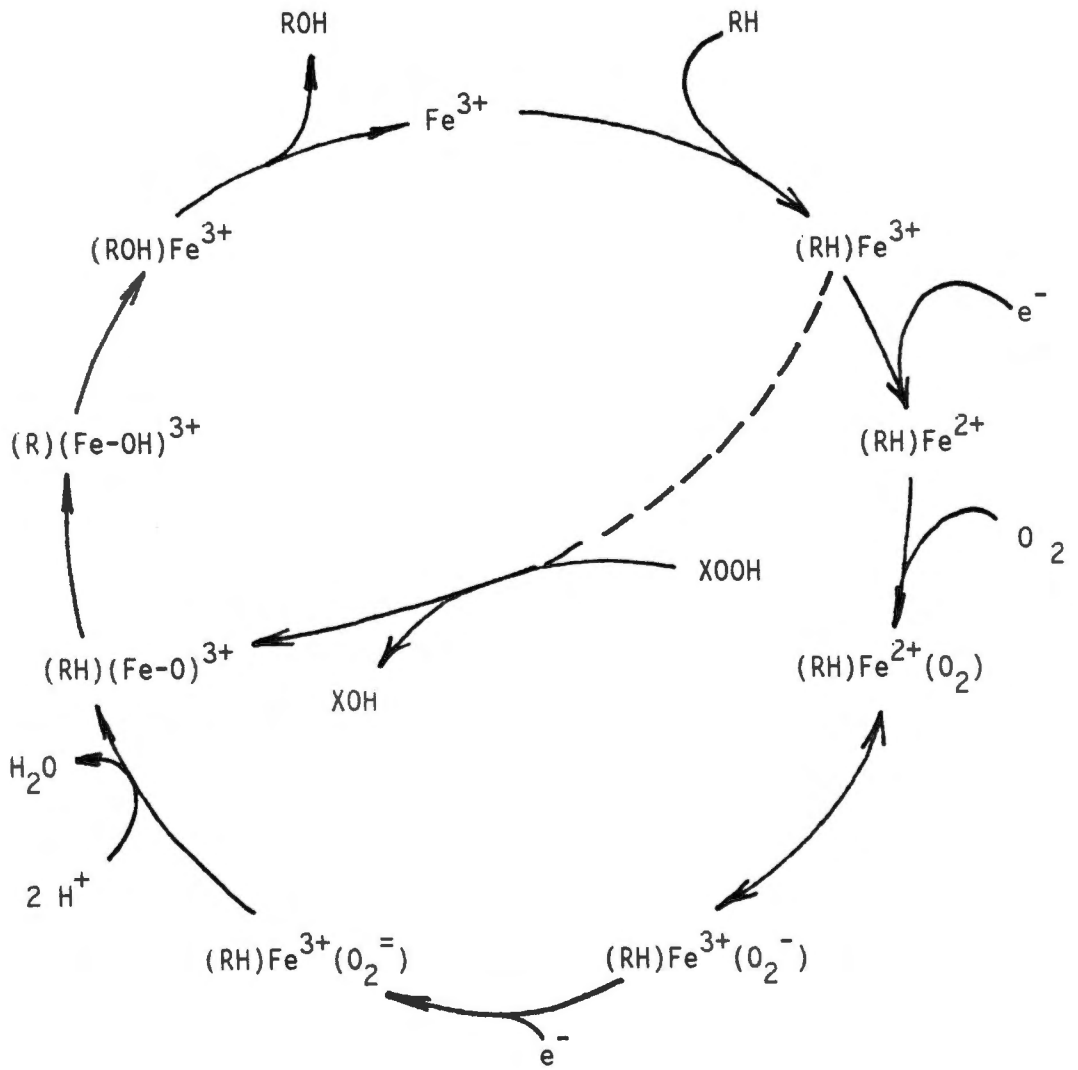


Figure 2. Proposed mechanism of action of liver microsomal cytochrome P-450, where RH represents the substrate, ROH the product, XOOH an oxidizing agent such as hydroperoxide or peracid, and XOH the corresponding alcohol or acid.

From Coon. 1978. Nutr. Rev. 36:324.

weights and substrate specificities (Guengerich, 1977). These isozymes have the ability to metabolize many of the same substrates but differ in their relative efficiencies (Coon, 1978).

Several nutritional factors including proteins, carbohydrates, fats, minerals and vitamins affect the activity of the MFO. Diets restricted in calories, proteins, or essential fatty acids affect the component enzymes (P-450 and P-450 reductase) and activity toward a variety of drugs (Bidlack et al., 1986). In addition, specific vitamins and minerals as well as certain drugs affect individual components and activities of the system. Therefore, insight into the regulation of MFO activity in ruminants through nutritional manipulation could prove invaluable in energy conservation.

Effect of Age

There is considerable evidence which demonstrates an age-dependent decline in the rate of drug metabolism (Schmucker, 1978). However, available information concerning specific effects of aging on drug metabolism and the mechanisms involved is extremely limited (Schmucker and Wang, 1980). Several factors may contribute to this problem including changes in intestinal absorption of drugs, body composition, hepatic drug metabolism and renal and hepatic clearance of drugs and their metabolites (Schmucker and Wang, 1980). Kato et al. (1964) reported that chronological age of rats and activities of their hepatic P-450 dependent drug-metabolizing enzymes were inversely related. Schmucker and Wang (1980) demonstrated a significant age-dependent decline in the concentration of hepatic smooth endoplasmic reticulum (SER) between maturity (16 months) and senescence (30

months) in Fischer 344 rats. In addition, significant age-related declines in P-450, P-450 reductase and ethylmorphine N-demethylase were also seen between maturity and senescence. This suggests a possible correlation between age-related changes in the quantity of hepatic SER and its constituent enzymes and hemeproteins (Schmucker and Wang, 1980). Unfortunately, information concerning the MFO system in ruminants is quite limited.

Changes in the Development of the Rumen

As the ruminant matures, the relative size of the abomasum decreases as the relative size of the rumen increases. Accompanying this change is variation of microorganisms present in the rumen (Church, 1970). Metabolites from microorganisms along with age of the animal may affect basal activity of the MFO (Burley and Bray, 1983). Since ruminants are subjected to numerous drugs and chemicals (e.g. antibiotics and pesticides) the activity of the MFO determines the rate of metabolism, excretion and residual time that these compounds remain in animal tissues (Burley and Bray, 1983). To study the effects of age on MFO changes in ruminants, goats were used as a model animal. Newborn goats (<1 day) exhibited lower hepatic microsomal protein and P-450 content than weanling (8-10 weeks) or adult (3-6 years) goats (Burley and Bray, 1983). However, no differences were observed between weanling and adult goats in either microsomal protein or P-450 content.

Effect of Drugs

Like dietary influences, various drugs have been shown to alter

MFO activity. Phenobarbitone induces proliferation of the smooth endoplasmic reticulum of the liver parenchymal cells and synthesis of several membrane-bound enzymes such as P-450, P-450 reductase and UDP-glucuronyltransferase (Davidson and Wills, 1974). More importantly phenobarbitone increases the linoleic acid proportion in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) by 120-125%. Increased activity of the MFO and concentrations of components correspond almost exactly to the increased incorporation of linoleic acid in PC and PE (Davidson and Wills, 1974). Davidson and Wills postulate that phosholipids of specific structure play an essential functional role in oxidative demethylation through membrane structure regulation and influencing bonds between proteins and phospholipids.

Phenobarbital (PB) has also been shown to infuence hepatic MFO activity. PB administration increases microsomal protein and P-450 content in newborn, weanling and adult goats (Burley and Bray, 1983) and P-450 reductase in rats (Marselos and Laitinen, 1975). Thomford et al. (1983) demonstrated increased microsomal protein, P-450, P-450 reductase, and cytochrome b5 (b5) in ewes dosed orally with 1 gram of PB daily which also resulted in increased ovulations (1.87 vs 1.61). Increased ovulations were probably a result of changes in estrogen production and metabolism through altered MFO activity.

Effect of Plant Substances

It has been suggested that the MFO plays an important role in the feeding strategies of herbivores (Brattsten and Wilkinson, 1977). Ehrlich and Raven (1964) proposed that development of these enzymes may have been forced by exposure of herbivores to the so-called

secondary substances of plants. In support of this, Krieg et al. (1971) demonstrated a correlation between polyphagy and high MFO activity.

Experiments conducted on the southern army worm moth (Spodoptera eridania) during its larvae stage gives support to these hypotheses. Compounds such as terpenoids, N-heterocyclics, glycosides and steroids enhance MFO activity from between 119-386% (Brattsten and Wilkinson, 1977). Very low concentrations of these compounds stimulate MFO activity and response to them was graded rather than "all-or-none." Brattsten and Wilkinson (1977) also demonstrated that larvae pretreated with terpenoids had lower susceptibility to nicotine poisioning than non-treated controls.

Effects of Protein

Nutritional status of an individual and the availability of nutrients are important regulators of xenobiotic metabolism (Bidlack et al., 1986). Many studies have shown that the protein/carbohydrate ratio affects drug metabolism. Decreasing the quality or quantity of protein decreases P-450, P-450 reductase and metabolism of ethylmorphine and aniline (Campbell and Hayes, 1976; Heitanen, 1980). The synthesis of cofactors such as 3'-phosphoadenosine-5'-phosphosulfate (PAPS) and glutathione (GSH) have also been shown to decrease during periods of protein deficiency (Bidlack et al., 1986). Anderson et al. (1979; 1982) demonstrated that isocaloric exchange of proteins for carbohydrates and lipids substantially shortens the plasma half-lives of antipyrine and theophylline, two compounds extensively metabolized by the liver. Conney et al. (1977) reported a

41% and a 36% decrease in plasma half-lives of antipyrine and theophylline, respectively, when subjects were changed from their normal home diets to diets containing high protein and low carbohydrate. Conversely, the average antipyrine and theophylline half-lives increased 63% and 43% respectively when subjects were changed from a high protein-low carbohydrate diet (44% protein, 35% carbohydrate, 21% fat) to a high carbohydrate-low protein diet (10% protein, 70% carbohydrate, 20% fat).

Studies conducted on rats indicate that a protein-deficient diet (5% protein) significantly lowers P-450 content (75%) and P-450 reductase activity (63%) when compared to controls receiving a 20% protein diet (Campbell, 1977). Kato et al. (1962; 1968) and Marshall and McLean (1969) also demonstrated similar results.

Woodcock and Wood (1971) reported that immature and mature rats fed a protein deficient diet (0% protein) had higher microsomal UDP-glucuronyltransferase activity compared to controls receiving an 18% protein diet. However, no change in sulfotransferase activity was observed.

These reports indicate that the individual component parts of the MFO are affected individually and in various ways, rather than as a unit.

Effect of Minerals

The active site of P-450 contains an iron protoporphyrin IX moiety within the hydrophobic cleft of the apoprotein (White and Coon, 1980). The heme is loosely bound by a combination of hydrophobic forces, Coulombic attractions and one or two coordinate-covalent bonds

to the central metal iron (White and Coon, 1980). Therefore, iron is essential for heme protein synthesis and maintenance of heme integrity.

Symes et al. (1969) showed that iron deficiency in rats leads to decreased hepatic iron levels and liver monoamine oxidase activity. Becking (1972) demonstrated no changes in P-450 or b5 content, however, P-450 reductase was significantly indreased in iron-deficient rats. In vitro and in vivo metabolism of aniline and aminopyrine were also increased in iron-deficient rats but only when hematocrit levels had decreased to approximately 65% of controls (Becking, 1972). Modification of P-450 properties with respect to substrate binding and/or rate of reduction of the P-450 substrate complex were offered as possible explanations for increased metabolism of aniline and aminopyrine during iron deficiency (Becking, 1972). Rats fed a magnesium deficient diet had lowered hepatic P-450 concentrations which coincided with reduced rates of aminopyrine metabolism in vitro and lowered rates of aniline metabolism both in vitro and in vivo (Becking and Morrison, 1970). In vito oxidation of PB and p-nitrobenzoic acid was not altered however.

Zinc deficiency in rats produces similar results to that of magnesium deficiencies including decreased P-450 concentrations and reduced metabolism of aminopyrine (Becking and Morrison, 1970). However, unlike magnesium deficiency, metabolism of PB and p-nitrobenzoic acid were also increased. Becking and Morrison (1970) theorize that binding of certain drugs to various P-450 isozymes, or alteration of heme protein synthesis is affected during periods of

magnesium and zinc deficiencies. However, selenium content of the diet had no effect on basal levels of P-450, b5 and P-450 reductase (Burke et al., 1974). PB administration increased P-450 reductase regardless of selenium status, however, P-450 and b5 failed to respond to PB treatment. Burke et al. (1974) concluded that selenium was necessary for hepatic heme synthesis, intracellular heme transport or for attachment of the heme to the apoprotein moiety of these cytochromes.

Effect of Maillard Reaction Products

The Maillard reaction is known to cause serious deterioration of food quality during both processing and storage (Nesheim and Carpenter, 1967; Tanaka et al., 1975). It is well known that amino acid destruction occurs during the Maillard reaction. However, amino acid destruction does not account for all of the impaired performance of animals fed browned products which suggests inhibitory or antinutritional compounds may be formed during the Maillard reaction. Although Kimiagar et al. (1980) did not measure MFO activity directly (i.e. P-450, P-450 reductase) several parameters which might indicate increased MFO activity, including serum alkaline phosphatase (SAP) and serum glutamic oxalic transaminase (SGOT) were determined. After 1 year of feeding a browned egg albumin diet SAP and SGOT activities were 130% and 40% higher, respectively, in rats fed the browned diet compared to controls (Kimiagar et al., 1980). In addition, reductions in weight gains and hematocrits and increases in liver weights also suggest possible effects on MFO activity as these parameters have been shown to behave in a similar fashion during increased MFO activity.

## Review of the Maillard Reaction

Three types of browning reactions occur in foods. First, oxidative reactions convert ascorbic acid and polyphenols into di- or polycarbonyl compounds. Secondly, caramelization, occurs when polyhydroxycarbonyl compounds are heated in the absence of amino compounds. And thirdly, the Maillard reaction, which is the most common, results when aldehydes, ketones, and reducing sugars react with amines, amino acids, peptides and proteins (Hodge, 1953). Maillard was the first to describe it systematically and percieve its biological consequences which included sugar and protein loss, formation of antinutrional or toxic substances and production of products with appetizing aromas (Adrian, 1974). Three characteristic steps occur during the Maillard reaction and are outlined by Hodge (1953). During the initial colorless stage sugar-amine condensation occurs with immediate Amadori rearrangement (see Figure 3). The second stage includes sugar dehydration, sugar fermentation and amino acid degradation, the products of which are termed premelanoidins. The final highly colored stage includes aldol condensation with final aldehyde-amine polymerization. The rate and pattern of these reactions are determined by the nature of the reacting amino groups and carbohydrates (DeMan, 1980). In addition, the intensity of browning can be enhanced further by heating, pH and moisture.

## Maillard Reaction in Forages

Heating in forages occurs through plant respiration, microbial metabolism and chemical reactions (Koegel and Bruhn, 1971) and the amount of water and oxygen within the forage mass determines the

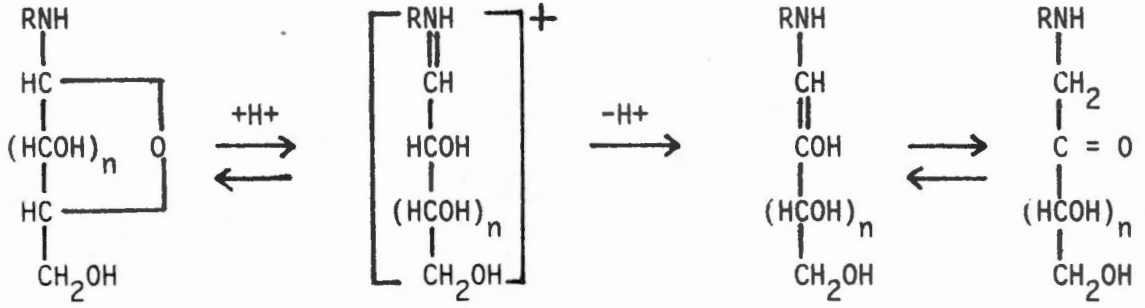


Figure 3. Steps showing the isomerization of N-substituted aldosylamines to 1-amino-1-deoxy-2-ketoses during Amadori rearrangement.

From Hodge. 1953. J. Agr. Food Chem. 1:928.

magnitude of these reactions (Wiess et al., 1986). Van Soest (1965) determined that heating of forages results in accumulation of a dark-colored nitrogenous polymer in the lignin fraction of acid detergent insoluble fiber (ADF). Van Soest (1982) also determined that the dark-colored polymer contained approximately 11% nitrogen (N) and possessed many of the same physical properties of lignin. Heating does not change the total N content of forages (Goering et al., 1973, Murdoch, 1960), only its distribution within plant material and consequently its availability. Increases in acid detergent insoluble nitrogen (ADIN), ADF and lignin and decreases in hemicellulose are also the result of the Maillard reaction.

Effect on Aroma and Flavor

Under correct conditions the Maillard reaction is capable of producing appealing aromas. A beneficial aspect of favorable aroma formation is increased palatability and intake through stimulation of the psychosensory senses. However, in the case of insufficient or excessive Maillard reactions unappealing aromas may form (Adrian, 1974).

Effect on Protein Availability

Ensiling of forages at lower moisture contents has increased the frequency of heat damage (Hill et al., 1963, Roffler et al., 1967) resulting in decreased apparent protein digestibility and animal performance when animals were fed heated forages (Donoso et al., 1962; Hill et al., 1963). Animals fed heat-damaged forages usually have lowered N balance (Miller et al., 1967; Yu and Thomas, 1975) and

apparent N digestibility (Wiess et al., 1986). Wiess et al. (1986) determined that the true digestibility of N in heated forages was 84% that of unheated forage and that concentrations of total amino acids in orchardgrass stored at 50% dry matter (DM) decreased linearly at the rate of 1.64 mg/g/day (Wiess et al., 1986).

Effect on Mineral Availability

When foods containing amino groups and reducing sugars are heated, Maillard products are formed which may range between early, water soluble, low-molecular-weight fractions to advanced, high-molecular-weight fractions (Lykken et al., 1986). Nair et al. (1981) demonstrated that up to 85% of the low-molecular-weight fractions are absorbed from the small intestine whereas the high-molecular-weight fractions were not absorbed. Therefore foods containing the high-molecular weight fractions may impair nutrient absorption as well. Freeman et al. (1975) and Stegink et al. (1981) observed excessive urinary zinc, copper and iron excretion when Maillard products in heat-sterilized parenteral solutions were administered intraveneously in humans. However, normal excretion patterns following nasogastric administration demonstrated that Maillard products evidently chelated these elements and rendered them unavailable when given intraveneously (Lykken et al., 1986). Human subjects fed browned cornflakes exhibited lower $^{65}Zn$ absorption compared to controls which were fed a non-browned corngrit diet (Lykken et al., 1986). Therefore, trace element bioavailability and retention are affected by Maillard products (Lykken et al., 1986).

## Possible Toxic Effects of the Maillard Reaction

Premelanoidins formed during the Maillard reaction are not only antinutritional but toxic as well. Mixtures of amino acids and glucose before heating show a lethal dose (LD50) range from 20 g/kg rat weight. After 10 min of heating, toxicity increases and the LD50 ranges from 4.1 to 11.2 g/kg depending upon the type of amino acids (Adrian, 1974). Premelanoidins given to rats during pregnancy result in lower maternal weight gains (27%), higher fetal resorption (5x), lower birth weights and fewer offspring (Adrian, 1974). Rats fed a browned egg albumin diet had significantly larger kidneys (34%) than controls after 6 months of feeding and after 12 months livers (21%), lung (16%), cecum (82%) and stomach (45%) also became significantly enlarged (Kimiagar et al., 1980). Abnormalities in kidney function were suspected in rats fed browned egg albumin due to higher urine specific gravity and enlarged kidneys (Kimiagar et al., 1980). Also histopathological examination of livers revealed the accumulation of a black-brown pigment, possibly hemosiderin, also suggesting physiological damage (Kimiagar et al., 1980).

## Summary

The mixed function oxidase (MFO, P-450) system is the primary drug metabolizing and detoxication system in the body (Bidlack et al., 1986). In ruminants, as well as nonruminants, activity of the MFO determines the rate of metabolism, excretion, and residual time that chemicals or toxins remain in animal tissues (Burley and Bray, 1983). Mixed function oxidases are essential for herbivores, due to intake of the so-called secondary substances of plants, e.g. compounds such as

phenols, quinones, terpenoids and alkyloids (Brattsten and Wilkenson, 1977). Phenolic compounds are also produced as a result of the Maillard or nonenzymatic browning reaction. Since the MFO has been identified as an energy wasteful system (Madsen and Miller, 1982; Pirola and Lieber, 1975; Smith, 1981) simulation by these compounds may reduce efficiency of enery utilization. Therefore, studies determining the effects of nonenzymaticaaly browned hay on MFO activity were conducted.

# CHAPTER III

## MATERIALS AND METHODS

Effects of age and nutrition on MFO activity were examined in one preliminary comparison with rats and three experiments with holstein steer calves.

### Preliminary Experiment

Eight male and eight female rats were selected from two litters from a closed colony maintained by the Department of Animal Science. After weaning they were fed Purina Rat Chow until the females and males weighed approximately 130 and 240 grams, respectively. The rats were then assigned to two groups, each containing two males and two females from each litter. The two groups were assigned to two dietary treatments: a commercially prepared semipurified diet (United States Biochemical, Clevland, OH) or the same diet with 5% added browned egg albumin. The diets were not isonitrogenous and probably not isocaloric due to the added browned egg albumin. However, the Maillard reaction probably reduced availability of the browned protein to negligible levels. Water and feed were supplied ad libidum to individually caged rats for 31 days.

### Preparation of Browned Egg Albumin

Browned egg albumin was prepared as described by Kimiagar et al. (1980). Egg albumin powder (U.S.B., Clevland, OH) and anhydrous D-dextrose (U.S.B., Clevland, OH) were combined in a 3:2 ratio with distilled water added to give a moisture content of 28%. The mixture

was kept in a sealed plastic container and placed in a sealed 37$^{o}$ water bath for ten days. A beaker containing a 40% sulfuric acid solution was also placed in the water bath to maintain a relative humidity inside the chamber at approximately 68%. The final product was freeze-dried and ground through a 2mm screen in a Wiley Mill.

Measurements

Rats were weighed prior to the start of the experiment and weekly thereafter. Feed was weighed every other day and amounts of refusals were recorded. On two consecutive days two males and two females fed each diet were weighed and sacrificed by stunning and decapitation. Wet weights of heart, liver and kidney were obtained immediately after sacrifice and livers placed in an iced beaker containing cold 1.15% KCL. Livers were then homogenized and assayed for cytochrome P-450 via a modified method of Omura and Sato (1964) (Appendix 1) and microsomal protein as described by Ohnishi and Barr (1978) (Appendix 2).

Calf Experiments

Holstein steers were used in three experiments which included effects of age, heat-browned hay, and amount of dietary protein on MFO activity. Calves were obtained from The University of Tennessee dairy herd at 2 d of age and housed in individual pens in a calf barn for 4 mo. They were fed a commercial milk replacer until 4 wk of age and offered hay and concentrate beginning the second week. Experiment 1 was conducted during the first 4 mo. Calves were then moved to a tie-stall barn for two additional experiments. Feed bunks were

partitioned to restrict each animal to its own ration. Water was available at all times through automatic water bowls. Calves were allowed 3 to 5 d to adjust to the new rations before each experiment.

Dosing and Measurement of Antipyrine

Activity of the MFO was assessed by measuring the rate of metabolism (disappearance from blood plasma) of antipyrine (2,3-dimethyl-1-phenyl-3-pyrozolin-5-one, Sigma Chemical Company, St. Louis, MO), a compound extensively metabolized by the liver (Anderson et al., 1982). Calves were dosed via a jugular vein with saline solutions of 18 mg antipyrine per kg body weight. Jugular blood samples were taken from the opposite vein at 0, 0.5, 1, 2, 3, 4 and 5 hr post dosing and kept in an ice bath until plasma was separated by centrifugation within 2 h. Antipyrine concentrations were determined spectrophotometrically (Bauch and Lomb Spectronic 2000) by the method of Brodie et al. (1949) (Appendix 3).

Experiment 1. Effect of Age on MFO Activity

Clearance of intraveneously administered antipyrine was measured in ten calves at 1, 2, 3, and 4 wks of age. Milk replacer feeding was discontinued the day before the first measurement was made. Calves were fed only concentrate and hay (clover-grass mixed) during the remainding 3 measurements.

Experiment 2. Effect of Heat-Browned Hay on MFO Activity

Two large round bales of alfalfa-grass hay were obtained from the Middle Tennessee Experiment Station at Spring Hill. Although both bales were from the same cutting, one was severly heat-browned whereas

the other was green colored and well cured. Acid detergent insoluble nitrogen (ADIN) contents were 41 and 9% of total nitrogen (N) in the heat-browned and well cured bales, respectively. The two hays were fed to two groups of five steers each. Antipyrine clearance rates were measured in a preliminary period during which all calves were fed the control hay (well cured) and again after the two hays had been fed for 1 and 2 wk.

Experiment 3. Effect of Dietary Protein Intake on MFO Activity

Twelve steers were utilized in a 2 X 2 factorial arrangement to compare effects on MFO activity of feeding two levels of supplemental protein with heat browned or control hay. Animals were assigned to one of four treatments minimizing weight differences between treatments. Animals averaged 215 kg and ranged from 150 to 255 kg. The four treatments were a low (13.3%) and a high (20.5) level of protein within the control and browned hay groups. Hay, corn and soybean meal (SBM) ratios for low and high protein diets were 75:25:0 (trt 1) or 75:7:18 (trt 2) for control hay, respectively, and 75:18:7 (trt 3) or 75:0:25 (trt 4) for heat-browned hay, respectively. Two large round bales of alfalfa-grass hay were again obtained from the Middle Tennessee Experiment Station at Spring Hill. ADIN contents were 13 and 18% of the total N in the control and browned hays, respectively. Antipyrine clearance rates were measured in a preliminary period during which all calves were fed the control hay and again after the two hays had been fed for 1 and 2 wk.

Statistical Analysis

Data were statistically analyzed using the regression (REG) and general linear models (GLM) procedure prepared by SAS institute (1985).

The data obtained from the rat experiment was analyzed by the GLM procedure according to the model:

$$Y_i = U + D_i + e_i$$

$Y_i$ = dependent variables

$U$ = theoretical population mean

$D_i$ = effect of diet
$i = 1\text{-}2$

$e_i$ = random error

Least square means were seperated using orthogonal contrasts (Sokal and Rohlf, 1981).

Calf data were analyzed in two steps. First a regression analysis was performed to determine regression coefficients (disappearance constants) using the following model:

$$Y_i = B_o + B_i + e_i$$

$Y_i$ = dependent variables

$B_o$ = intercept

$B_i$ = regression coefficient of the $i^{th}$ treatment

$X_i$ = value for the $i^{th}$ independent observation

$e_i$ = random error

Secondly, a GLM procedure was used to establish differences between regression coefficients. The following model was used:

$$Y_i = U + D_i + e_i$$

$Y_i$ = dependent variables

$U$ = theoretical population mean

$D_i$ = effect of diet

$e_i$ = random error

Least square means were seperated using orthogonal contrasts.

# CHAPTER IV

# RESULTS

## Preliminary Experiment

Results from the preliminary rat experiment are summarized in Table 1. Sex differences were observed in some but not all measurements. Males consumed more feed ($P < .10$) and had higher hepatic concentrations of cytochrome P-450 ($P < .05$) than females. There were no sex differences in heart and kidney weights. Weight gain or heart and kidney weights of both males and females did not differ between groups fed control or treated diets. However, control males consumed 8.5% more feed ($P < .10$) than their treated counterparts. When treated versus control diets are compared, females fed browned egg albumin exhibited heavier livers ($P < .06$); whereas there was no response in males. Conversely, males fed the browned diet displayed higher concentrations ($P < .05$) of P-450 (nmoles/mg protein) whereas females did not. This agrees with Kimiagar et al. (1980) who observed greater susceptibility to browned products by males than by females. Although the difference in female rats was not statistically significant, P-450 concentrations were over five times greater in those fed browned diets than in controls. In addition, P-450 concentrations of control females paralleled those of control males. Higher protein content of the browned diet could have contributed to increased P-450 concentrations if the added protein was biologically available.

Table 1. Effect of Maillard products on feed consumption, weight gain, heart, kidney and liver weights and cytochrome P-450 content in rats.

| | Males | | Females | |
|---|---|---|---|---|
| | Control | Treated | Control | Treated |
| FEED CONSUMED (total grams) | 664.80†** | 607.80** | 498.4 | 469.10 |
| WEIGHT GAIN (grams) | 112.00** | 95.50** | 45.00 | 46.50 |
| HEART WEIGHT (% B.W.) | 0.42 | 0.43 | 0.49 | 0.49 |
| KIDNEY WEIGHT (% B.W.) | 0.85 | 0.89 | 0.90 | 0.91 |
| LIVER WEIGHT (% B.W.) | 4.96* | 4.98 | 4.67 | 5.06† |
| CYTOCHROME P-450 (nmoles/mg protein) | 0.0765 | 0.2006†† | 0.0281 | 0.1429 |

†Significantly different at P<.06 (Within sex).
††Significantly different at P<.05 (Within sex).
*Significantly different at P<.10 (Within treatment across sex).
**Significantly different at P<.001 (Within treatment across sex).

Experiment 1. Effect of Age

The objective of this study was to compare MFO activity in calves at various ages. This comparison was necessary to determine whether age related changes in activity might contribute to possible increases in MFO activity in later experiments. Results of the effect of age on MFO activity are shown in Figure 4. Disappearance constants (mg/dl plasma $hr^{-1}$) were -1.019, -0.590, -0.699 and -0.634 for 1, 2, 3, and 4 mo of age. Calves metabolized antipyrine 72.7%, 45.8% and 60.7% faster ($P < .05$) at 1 mo than at 2, 3, or 4 mo, respectively. There were no differences in the rate of metabolism between 2, 3, and 4 mo.

Experiment 2. Effect of Heat-Browned Hay

Composition of the two hays can be found in Table 2. Nutrient composition of the two hays were similar except the percentage of N in ADIN was 4.8 times higher in the heat-browned hay than in the control hay (8.47 vs 41.22%). Results of this experiment are found in Figures 5 and 6. Due to this marked difference in ADIN content available protein in the control and heat-browned hays was 15.56 and 9.39%, respectively. Preliminary disappearance rates of antipyrine (mg/dl plasma $hr^{-1}$) were similar between the two groups of calves later fed control ( -0.508) or heat-browned ( -0.462) hay. Control animals cleared antipyrine at a similar rate (-0.487) than animals fed the heat-browned hay (-0.510), however, differences were not significant. The experiment was carried out an additional week but no changes were observed. Clearance rates for the control and heat-browned groups were -0.459 and -0.514, respectively.

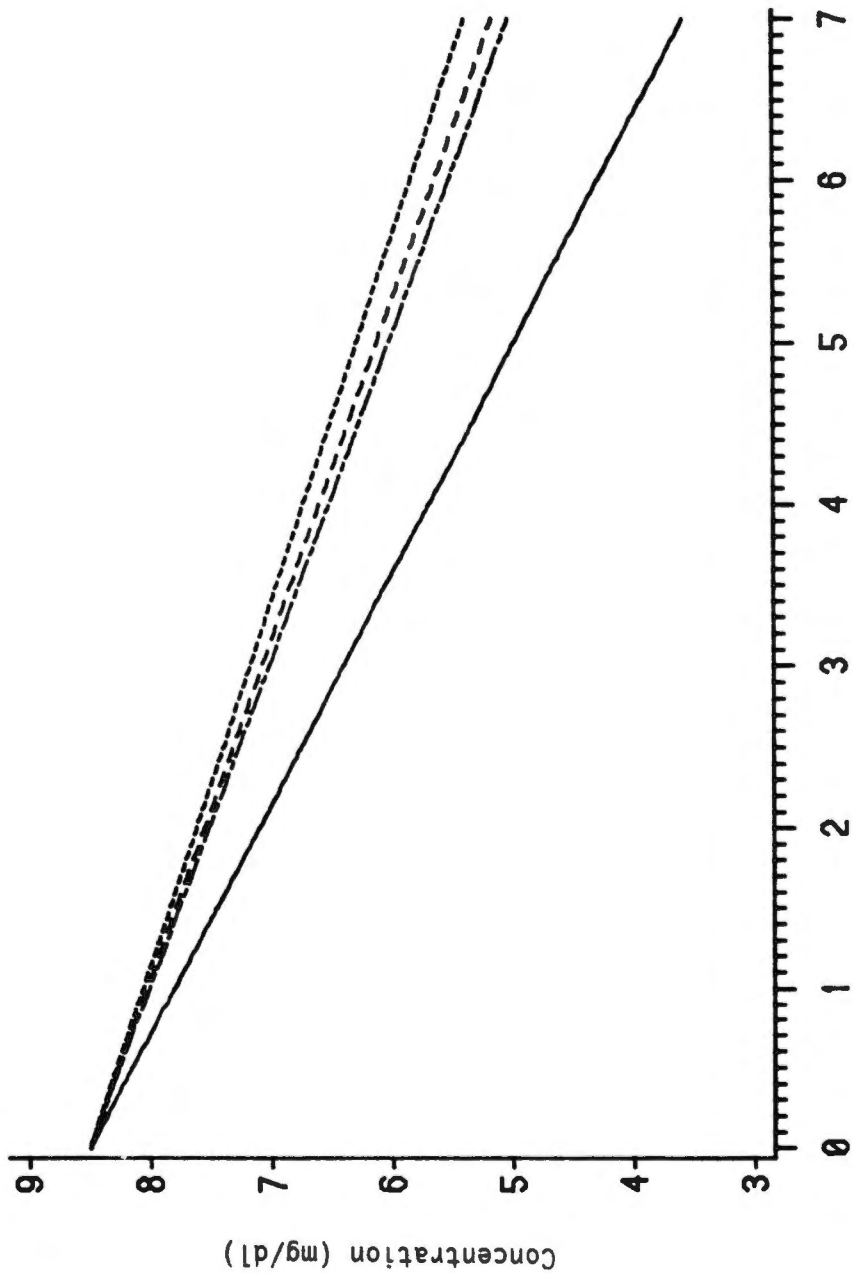


Figure 4. Effect of age (months) on antipyrine disappearance in calves.

Table 2. Composition of control and heat-browned hays in calf experiment 2.

| Nutrient | Control | Heat-Browned |
|---|---|---|
| | ----------(%)---------- | |
| Dry matter | 96.16 | 95.77 |
| | ------(% of dry matter)------ | |
| Crude protein | 17.00 | 15.97 |
| Crude fiber | 34.57 | 27.76 |
| Ash | 8.30 | 6.84 |
| Ether extract | 2.83 | 2.44 |
| Nitrogen free extract | 51.58 | 60.41 |
| Acid detergent fiber | 40.31 | 41.54 |
| | ----(% of total nitrogen)---- | |
| Acid detergent insoluble nitrogen | 8.47 | 41.22 |

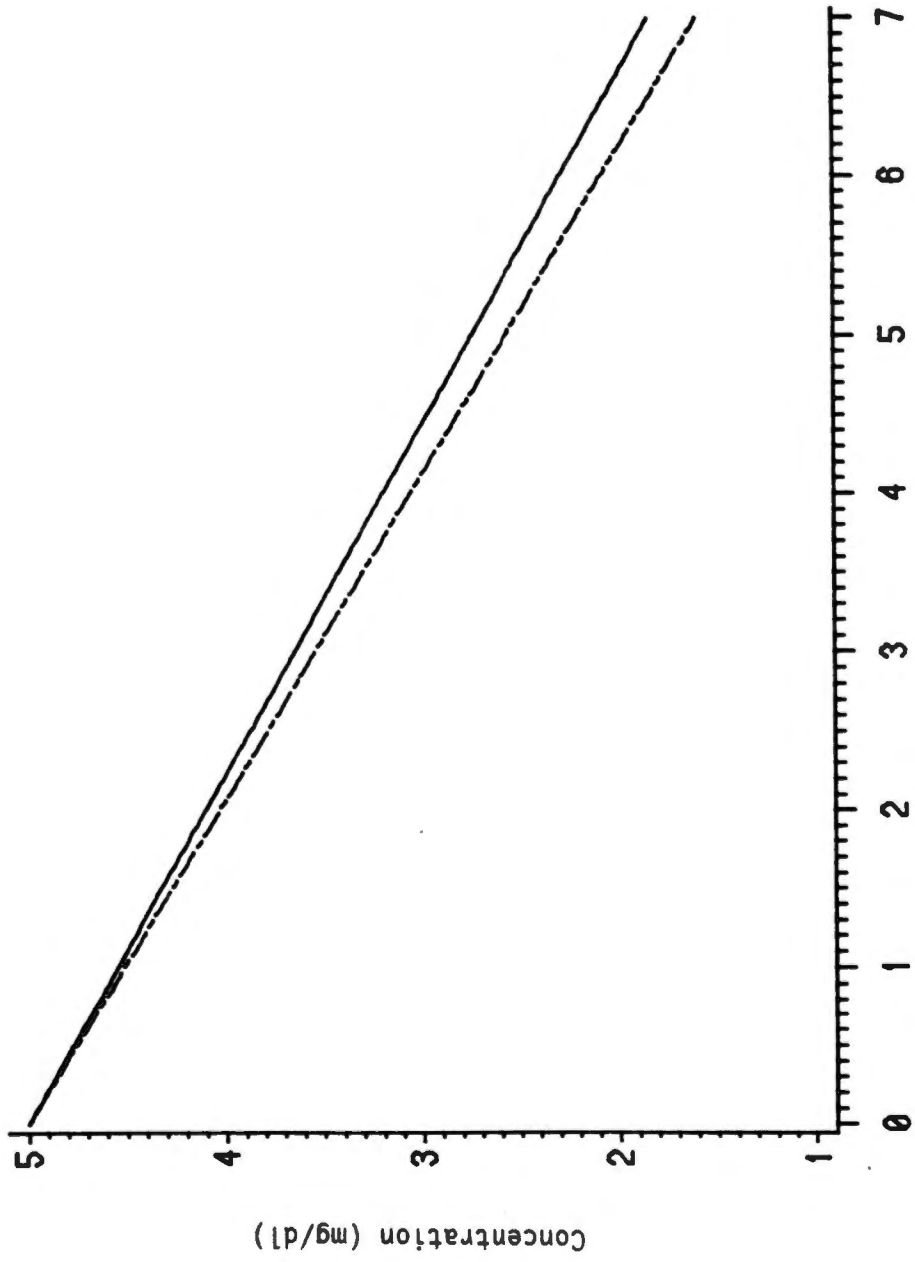


Figure 5. Effect of heat-browned hay on antipyrine disappearance in calves (Week 1).

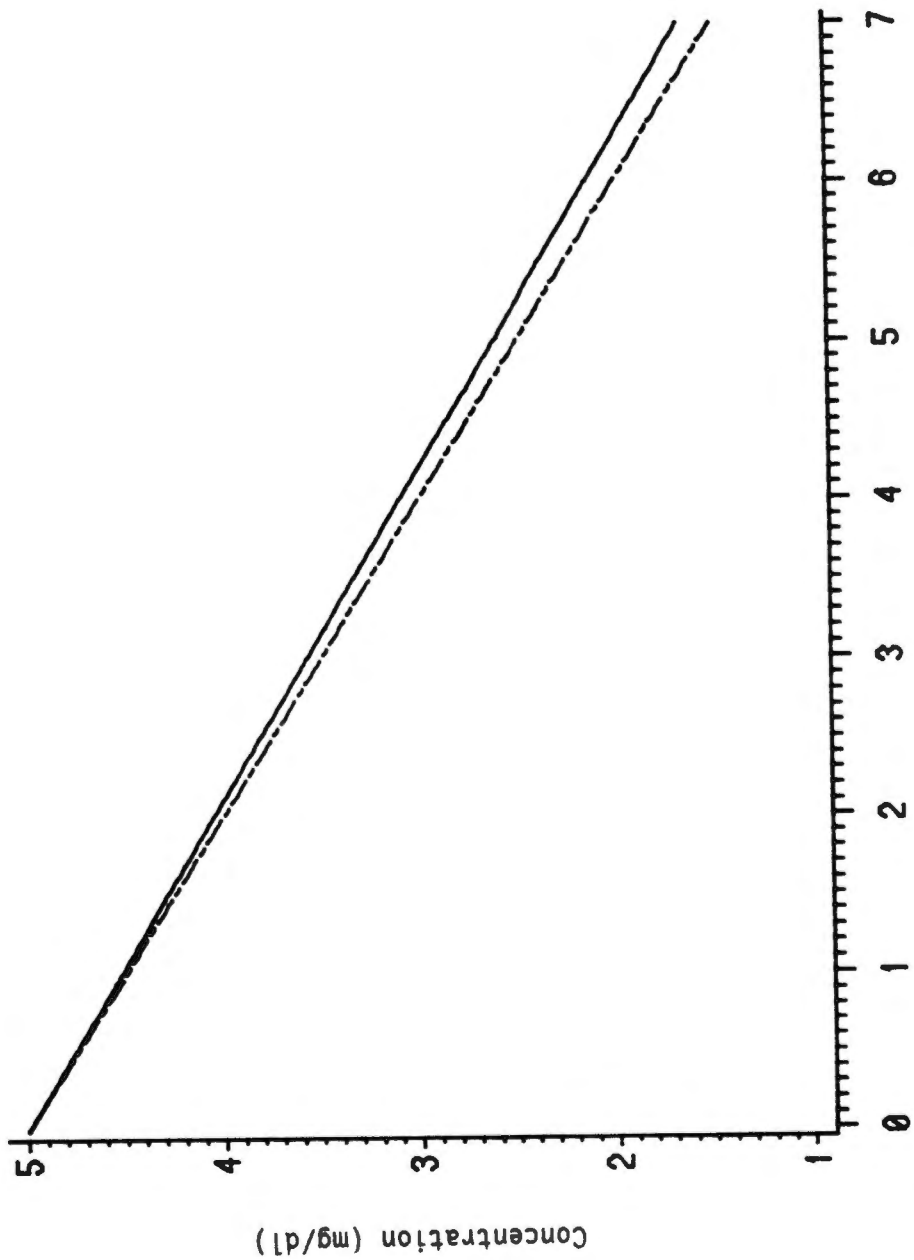


Figure 6. Effect of heat-browned hay on antipyrine disappearance in calves (Week 2).

Experiment 3. Effect of Dietary Protein

Composition of hay, protein levels and ratios of hay, corn and SBM are summarized in Tables 3 and 4. Nutrient content of browned hay was similar to that of control hay except crude protein was 3.62% lower and ADIN was 5.59% higher. The four treatments consisted of a low (13.3%) and a high (20.5%) level of protein. Ratios of hay, corn and SBM for low and high protein diets were 75:25:0 (trt 1) and 75:7:18 (trt 2), respectively, for the control hay and 75:18:7 (trt 3) or 75:0:25 (trt 4), respectively, for the heat-browned hay.

Results are shown in Figure 7. Preliminary disappearance rates of antipyrine (mg/dl plasma $hr^{-1}$) were similar between the four treatment groups later fed the control hay-low protein (-0.721), control hay-high protein (-0.722), browned hay-low protein (-0.430) and browned hay-high protein (-0.728). After 1 wk of feeding diets disappearance rates of groups fed treatment 1, 2, 3 and 4 were -0.632, -0.489, -0.632 and -0.646, repectively. No significant differences were observed, however, a general tendency toward slower rates of disappearance than in the preliminary measurement was observed. Similarly, after 2 wk of feeding diets disappearance rates were -0.612, -0.538, -0.605 and -0.510, respectively, for treatments 1, 2, 3 and 4. Disappearance rates from the 2 wk were generally lower than 1 wk observations, however, the difference was not statistically significant.

Table 3. Composition of control and heat-browned hays in calf experiment 3.

| Nutrient | Control | Heat-Browned |
|---|---|---|
| | ----------(%)---------- | |
| Dry matter | 91.56 | 90.39 |
| | --------(% of dry matter)------ | |
| Crude protein | 16.55 | 13.26 |
| Crude fiber | 36.61 | 34.18 |
| Ash | 8.24 | 6.60 |
| Ether extract | 2.16 | 2.11 |
| Nitrogen free extract | 50.36 | 55.01 |
| Acid detergent fiber | 43.37 | 41.34 |
| | ----(% of total nitrogen)---- | |
| Acid detergent insoluble nitrogen | 12.58 | 18.17 |

Table 4. Dietary protein percentage (available protein) of the four treatments in calf experiment 3.

| | Control Hay | Heat-Browned Hay |
|---|---|---|
| | (%) | |
| Low protein | 13.37 | 13.43 |
| High protein | 20.49 | 20.53 |

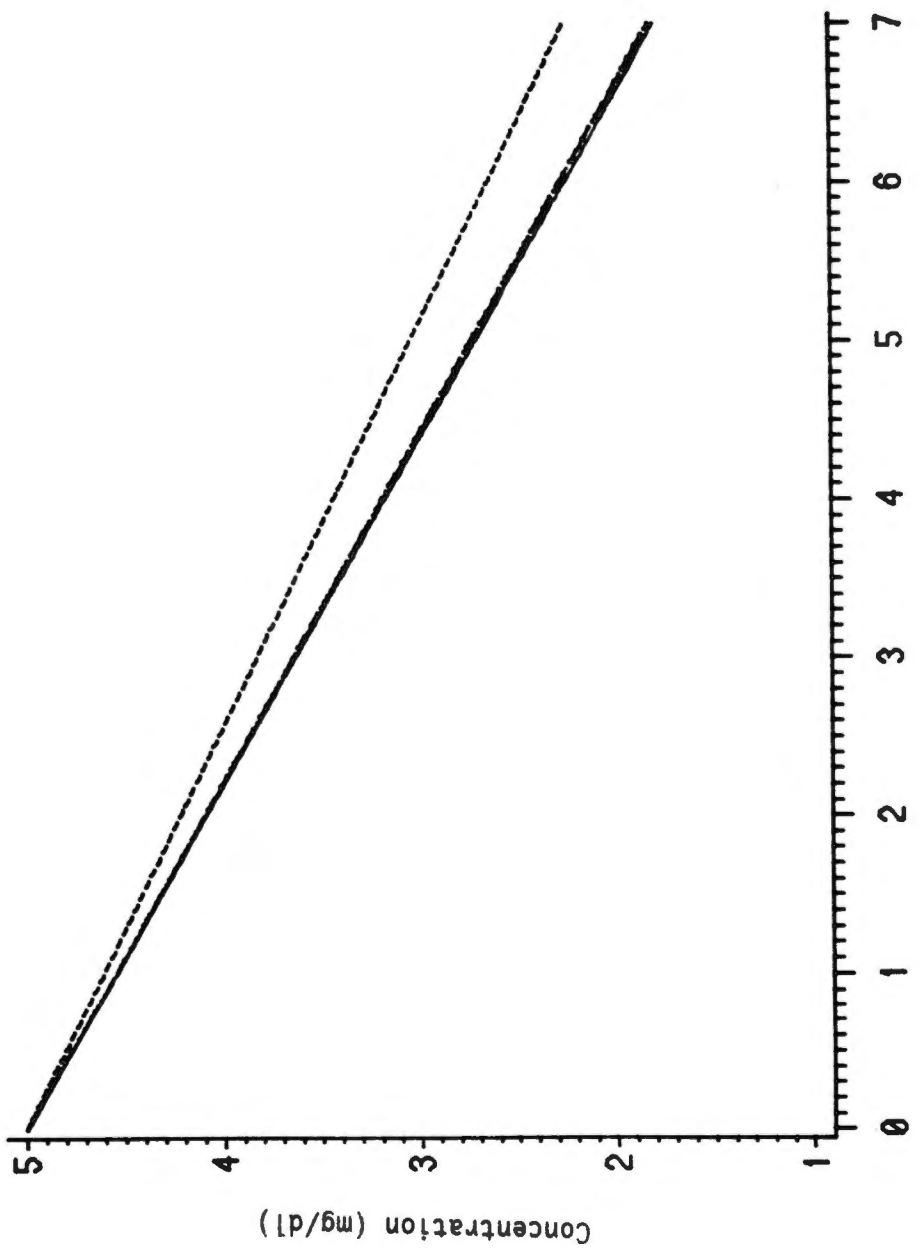


Figure 7. Effect of dietary protein on antipyrine disappearance in calves.

# CHAPTER V

# DISCUSSION

## Preliminary Experiment

Detrimental effects of feeding Maillard productson animal performance and health have been recognized for years. Long-term feeding of Maillard products to rats results in physiological damage to kidneys and livers as indicated by significantly higher specific gravity of urine and increased activities of SAP and SGOT. Adverse affects of Maillard products increase with length of time they are fed. Noticeable changes were seen within 31 d in rats fed browned egg albumin (Table 1, page 27). Male rats fed browned diets consumed 7% more feed per unit of weight gain and also exhibited 2.6 times higher concentrations of P-450 in liver than controls ($P < .05$). Enhanced MFO activity could have contributed to the reduced feed efficiency in treated males. Pirola and Lieber (1975) demonstrated that stimulation of inefficient microsomal enzyme systems (i.e. MFO) can significantly alter the body's metabolic efficiency. Treated females displayed 8.4% heavier livers ($P < .06$) and 5 fold greater concentrations of hepatic P-450 than controls but P-450 differences were not statistically significant. A lower response to Maillard products by females than by males agrees with results of Kimiagar et al. (1980) and may result from endogenous estrogen. Takahashi and Jensen (1984) found hepatic P-450 levels were reduced in chicks by administration of 8.5 ug of estradiol ($E^2$) per day. Degree of these changes were determined by estrogen dosage level and period of administration. Jellineck and

Harland (1969) also demonstrated marked reduction in $^{14}C$-$E_2$ metabolism as a result of lowered MFO activity in estrogen treated chicks.

Experiment 1. Effect of Age

There is considerable evidence demonstrating an age-dependent decline in the rate of drug disposition (Schmucker, 1979). Contributing factors include changes in drug absorption, body composition, hepatic metabolism and renal and hepatic clearance of drugs and their metabolites (Schmucker and Wang, 1980). In monogastrics digestive processes do not seem to alter metabolism of drugs and toxins since absorption occurs in the intestinal region. However, in the ruminant, MFO activity may be altered by changes within the rumen. During rumen development bacteria and protozoa populations vary with factors such as diet and age (Church, 1970). Rumen fermentation products also vary depending upon the substrate and species of microorganisms present. Unfortunately, the effects that these fermentation end-products have on MFO activity cannot be seperated from the effect of age (Burley and Bray, 1983). Burley and Bray (1983) found concentrations of P-450 in goat livers were 95% higher in weanling and 226% higher in adults than in newborns. Our study indicated that after 1 mo. of age rates of antipyrine metabolism decreased. At 2, 3 and 4 mo. metabolism rates were 72, 45 and 60% slower than at 1 mo. of age. During weaning gradual discontinuation of milk feeding is accompanied by gradual increases in dietary solid intake. Accompanying these changes is a decrease in dietary protein intake. Just prior to our 2nd mo. sample, calves were weaned. Changes in MFO activity could have resulted from a change in dietary

protein intake. Since normal changes in rumen development occur within the first 3 to 5 mo. of age separation of these effects from age is impossible.

Experiment 2. Effect of Heat-Browned Hay

Animals fed heat-browned feeds display lower N digestibilities (Bechtel et al., 1945; Miller et al., 1967) and N retentions (Donoso et al., 1962; Miller et al., 1967; Yu and Viera, 1977). Heating also reduces amino acid availability in forages (Wiess et al., 1986). Consequences of feeding heat-browned feeds is not limited to poor performance. Van Soest described a condition termed "bovine bonkers" in cattle fed heat-browned feeds for extended periods. Cattle roamed aimlessly, ran into objects and were extremely difficult to handle (personal communication). In addition, Madsen observed liver dysfunction in dairy herds fed heat-browned forage. Liver function returned to normal when browned forage was diluted with unbrowned forage (personal communication). In our study MFO activity was unaffected by heat-browned hay. After 2 wk. of feeding severely browned hay (ADIN 41%) MFO activity was similar between the control and browned hay groups. Available protein in the control and browned hays was 15.6 and 9.4%, respectively. Decreasing protein quality or quantity have been shown to reduce levels of P-450 and P-450 reductase (Campbell and Hayes, 1976; Heitenen, 1980). Reduced protein availability of the browned hay could have offset any effect that Maillard products might have had on MFO activity in our experiment.

Experiment 3. Effect of Dietary Protein

In experiment 2 differences in protein intake may have nullified any Maillard effects on antipyrine disappearance rates. Experiment 3 was designed to separate effects of dietary protein intake from Maillard products. The factorial design made it possible to compare treatments within and across hay diets. Results showed that neither the added protein nor heat-browned hay had any effect on antipyrine disappearance rates. Results from this experiment contradicted the results obtained in the preliminary study. Several reasons may account for these differences.

First, rats are monogastrics (nonruminants) whereas cattle are ruminants. Rate of food passage between ruminants and nonruminants differ. On the average, rates of passage for ruminants and nonruminants are 48 to 72 and 12 to 18 hrs., respectively. Our feeding studies were conducted over a period of 19 d allowing 3 to 5 d adjustment periods. Typically, nutritional studies conducted with ruminants allow for a 10 to 14 d adjustment period. The limited amount of control hay in our studies dictated the length of the trial and more so the length of the adjustment periods. Kimigar et al. (1980) determined that detrimental effects of Maillard products fed to rats appear by the 3$^{rd}$ mo. of feeding and increase the longer they are fed. We may not have seen differences in our hay experiments due to insufficient length of feeding. Long-term feeding (> 3-5 mo.) of heat-browned hay may be necessary to demonstrate effects on MFO activity in ruminants.

Secondly, a significant difference in digestive processes exists between ruminants and nonruminants. Microorganisms found in the rumen enable ruminants to obtain nutrients from feeds which are unavailable to nonruminants. More importantly, is the possible detoxication properties of these microorganisms. Detoxication may occur within the rumen to some extent before harmful substances are absorbed. Therefore, the MFO could play a smaller role in detoxication of ingested substances in the ruminant.

Thirdly, the effects of salivation must be considered. After these experiments were concluded an additional experiment was conducted on catheterized ewes. Animals were surgically prepared as described by Katz and Bergman (1969). Simultaneous blood samples drawn from the femoral artery and femoral, portal, hepatic and mesenteric veins permitted the calculation of tissue uptakes of antipyrine. Blood flows were calculated by measuring down stream dilution of p- aminohippuric acid and net fluxes were calculated by multiplying venoarterial differences by tissue blood flows (Sensenig, personal communication). Liver extraction of antipyrine only accounted for 10 to 20% of the initial dose. Since antipyrine equilibrates readily in body water and ruminants produce up to 200 l of saliva per day, antipyrine could have been carried by saliva to the rumen where it was finally metabolized.

## Conclusion

The preliminary experiment demonstrated that Maillard products increase cytochrome P-450 and therefore MFO activity. However, this response was not seen when steers were fed Maillard products. Several

reasons were given as possible explanations. From these studies it was concluded that antipyrine disappearance is not a suitable indicator of MFO activity in ruminants. In species that do not produce great volumes of saliva antipyrine disappearance is an accurate method of determining MFO activity.

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# APPENDICES

# APPENDIX 1

# DETERMINATION OF CYTOCHROME P-450

## Reagents

1. 1.15% cold KCL
2. Phosphate buffer 0.1M pH = 7.0
3. 15 mg $Na_2S_2O_4$/ml buffer

## Procedure

1. Homogenize livers with 4 volumes isotonic (1.15%) KCL.
2. Centrifuge at 12,000 x g for 25 minutes at $4^oC$.
3. Decant and centrifuge again at 12,000 x g for an additional 25 minutes at $4^oC$.
4. Centrifuge supernatant fluid at 100,000 x g for 60 minutes.
5. Resuspend microsomes in 1.15% KCL to equal about 10 mg protein/ml. Approximately 2.5 ml.
6. Add 10 ml of 0.1M $PO_4$ buffer pH 7.0 to suspension.

## Reading Absorbance

1. Set spectophotometer at 350 nm.
2. Place 3 ml of sample in cuvette and check absorbance. Make sure it falls between 0.5-0.7.
3. Bubble in CO for 1 minute and read.
4. Add 45 mg $Na_2S_2O_4$ and mix by transferring solution from the test tube to the cuvette 3 times to allow proper mixing.
5. Read absorbance.
6. Calculate the nanomoles of cytochrome P-450 using the extinction coefficient of 91 $cm^{-1}mM^{-1}$.
7. Determine protein concentration and express as nmoles/mg protein.

Standard Curve

1. Standards are prepared by placing 3 ml of a known solution of antipyrine in 0.07N Sulfuric Acid in a cuvette and reading the optical density before and after the addition of Sodium Nitrite.

2. 0.07N Sulfuric Acid similarly treated is used as a zero setting.

3. Standards are run with each set of determinations since there is a daily variation of about 3 percent in the optical density.

# APPENDIX 2

# DETERMINATION OF MICROSOMAL PROTEIN

## Reagent Preparation

1. Biuret

   Dissolve in 500 mls $DDH_2O$;

   1.5 gs $CuSO_4 * 5\ H_2O$

   6.0 gs $NaKC_4H_4O_6 * 4\ H_2O$

   Stirring constantly, add 300 mls of freshly prepared, carbonate-free 10% (w/v) NaOH (30 gms NaOH dissolved in 300 mls of preboiled $DDH_2O$ water)

   Dilute to 1 liter

2. 2.3% $Na_2CO_3$

3. Protein

   Mix the Biuret and 2.3% $Na_2CO_3$ reagents in a (1:7, v/v) ratio

## Procedure

1. Subcellular fractions were appropriatelty diluted to yield 50-600 ug protein per ml.
2. To 0.8 mls of sample were added 3.2 mls of the "protein reagent". Vortex and allow to stand for 10 min.
3. While vortexing the mixture again, 0.1 mls of undiluted 2N Folin-Ciocalteau phenol reagent was added. Allow mixture to stand for 30 min.
4. Absorbance was measured at 750 nm against a water blank.
5. A standard was prepared using bovine serum albumin. ( 160 ug / 0.8 mls )

# APPENDIX 3

# DETERMINATION OF ANTIPYRINE

## Reagents

1. Zinc

   Dissolve in 700 mls $DDH_2O$:

   100 gm of $ZnSO_4 * 7\ H_2O$

   40 ml 6N Sulfuric Acid

   Bring to 1 liter.

2. 0.75N Sodium Hydroxide

3. 4N Sulfuric acid

4. 0.2 percent Sodium Nitrite solution

## Procedure

1. To 2 ml of plasma add 2 ml of water and 2 ml of Zinc reagent.
2. Add 2 ml of 0.75N Sodium Hydroxide and vortex for 30 seconds.
3. Allow to stand for 10 minutes, then centrifuge at 7500 R.P.M. for 15 minutes.

## Reading Optical Density

1. Transfer 3 ml of clear supernatant to cuvetee and add 1 drop (25 ul) of 4N Sulfuric Acid.

2. Read optical density at 350 mu. 3 ml of water plus 25 ul 4N Sulfuric Acid are used for the zero setting.

3. Add 2 drops (50 ul) of 0.2 percent Sodium Nitrite solution to both the unknown and the zero setting and read the optical density again after 20 minutes.

4. Subtract from this the optical density obtained before the addition of the Nitrite.

# VITA

Kenneth Patrick Zanzalari was born in Perth Amboy, New Jersey, on April 8, 1961, to Mr. and Mrs. Robert M. Zanzalari. He was reared in Woodbridge, New Jersey and attended public school #11 and graduated from Woodbridge Senior High in 1979. The following September he attended Susquehanna University for one year and later transferred to Delaware Valley College of Science and Agriculture, Doylestown, Pa. He recieved his B.S. degree in Dairy Science from DVC in 1984. In September of 1984, he accepted the position of Graduate Research Assistant at The University of Tennessee, Knoxville, to begin his studies toward a Master's of Science degree in Animal Science, specializing in ruminant nutrition, in March 1987. The author is a member of Gamma Sigma Delta and the American Dairy Science Association.