Influence of dietary protein source and exogenous progesterone on liver mixed function oxidase activity in ovariectomized ewes

by Kathryn Jo Wiley

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Animal Science

Montana State University

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Abstract:

Nineteen ovo-hysterectomized Rambouillet ewes from 3 genetic lines resulting in different reproductive prolificacy (high-H, control-C, low-L) were assigned to one of two treatments: Urea (U) and Blood Meal (BM) . Diets were isocaloric and isonitrogenous and ewes were fed at 1.5 X maintenance for nonpregnant, non-lactating dry ewes. Liver biopsies were taken surgically before the trial (pre-trt) and 19d after trial had begun (post-trt) and analyzed for microsomal protein and cytochrome P-450. Progesterone was infused through jugular catheters and blood samples drawn over a period of 12 h. Blood samples were analyzed for progesterone and blood urea nitrogen (BUN). Initial and final live weights, dry matter and protein intake, and pre and post microsomal protein content were not different (P>.10). Pre-treatment cytochrome P-450 concentrations were not different (P>.10) between treatment groups. However, post-treatment cytochrome P-450 concentrations were different between U and BM (P<.05) with U group having greater concentration of cytochrome P-450 (1.3 vs .73 nmol mg-1 protein). Blood urea nitrogen concentration tended to be higher in U group (P=. 14) with 13.2 mg dl-1 in U and 8.2 mg dl-1 in SM. High line ewes tended to have greater P-450 concentrations than other lines (P>.10), which resulted in a significant change (P<.10) in P-450 concentrations in H compared to L and C. Quadratic regression on the progesterone indicated no difference between treatments for progesterone disappearance rates. These data suggest that protein quality influenced P-450 concentration. The P-450 concentrations appeared to be higher in ewes fed U that had been selected for high reproductive rates. Progesterone disappearance rates could possibly have been confounded since progesterone injection and sampling sites were the same.

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Nineteen ovo-hysterectomized Rambouillet ewes from 3 genetic lines resulting in different reproductive prolificacy (high-H, control-C, low-L) were assigned to one of two treatments: Urea (U) and Blood Meal (BM). Diets were isocaloric and isonitrogenous and ewes were fed at 1.5 X maintenance for non-pregnant, non-lactating dry ewes. Liver biopsies were taken surgically before the trial (pre-trt) and 19 d after trial had begun (post-trt) and analyzed for microsomal protein and cytochrome P-450. Progesterone was infused through jugular catheters and blood samples drawn over a period of 12 h. Blood samples were analyzed for progesterone and blood urea nitrogen (BUN). Initial and final live weights, dry matter and protein intake, and pre and post microsomal protein content were not different (P>.10). Pre-treatment cytochrome P-450 concentrations were not different (P>.10) between treatment groups. However, post-treatment cytochrome P-450 concentrations were different between U and BM (P<.05) with U group having greater concentration of cytochrome P-450 (1.3 vs .73 nmol mg^{-1} protein). Blood urea nitrogen concentration tended to be higher in U group (P=.14) with 13.2 mg dl^{-1} in U and 8.2 mg dl^{-1} in BM. High line ewes tended to have greater P-450 concentrations than other lines (P>.10), which resulted in a significant change (P<.10) in P-450 concentrations in H compared to L and C. Quadratic regression on the progesterone indicated no difference between treatments for progesterone disappearance rates. These data suggest that protein quality influenced P-450 concentration. The P-450 concentrations appeared to be higher in ewes fed U that had been selected for high reproductive rates. Progesterone disappearance rates could possibly have been confounded since progesterone injection and sampling sites were the same.

CHAPTER 1

INTRODUCTION

Cytochrome systems are complex enzyme systems found in most forms of living organisms. Since the early 1960's the majority of research has been to determine their location in different tissues and mode of action. Cytochrome systems play a key role in the nutritional/reproductive axis in ruminants. Cytochromes can be manipulated nutritionally and in turn, affect animal reproduction.

It has been shown that nutrition increases the concentration of the cytochrome systems in liver cells. In turn, these systems affect reproduction by influencing the concentrations of circulating hormones related to reproduction. (Thomford and Dziuk, 1986; Smith, 1988; Thomford and Dziuk, 1988). An example of this is the flushing effect in sheep. "Flushing" has long been used as a management tool to increase ovulation rate by subjecting the ewes to a high plane of nutrition three to four weeks prior to breeding. Smith (1988) indicated that quantity or quality of dietary protein fed may affect these cytochrome enzyme systems. However, increased plane of nutrition associated with flushing if continued after conception has occurred has a carryover

effect (Smith et al., 1983) which increases early embryo mortality in sheep (Williams and Cumming, 1982 Parr et al., 1982; Parr et al., 1987). However, the mechanism describing this phenomena has not been determined. Diskin et al., (1988) states that, "Embryo mortality is a major cause of economic loss in the sheep industry."

The objective of this study was to determine the influence of supplemental protein source on cytochrome systems and secondly, to determine whether clearance rate of progesterone from the blood increases with increased enzyme activity. These data may lead to a better understanding of how nutrition impacts reproduction in sheep.

CHAPTER 2

LITERATURE REVIEW

Cytochrome P-450

Cytochromes P-450 are found in the testis (Menard et al., 1973), adrenal glands (DuBois et al., 1981; Kramer et al., 1982 Ohashi et al., 1983), liver (Conney and Klutch, 1963; Omura and Sato 1964a; Omura and Sato 1964b; Kaminsky et al., 1981; Thomford and Dziuk, 1986; Thomford and Dziuk, 1988), kidney, lung, colon, heart, brain, spleen, small intestine, muscle (Kaminsky et al., 1981), and rumen papillae (Smith et al., 1983; Watkins et al., 1987). P-450 has also been found in ovaries and associated structures (Funkenstein et al., 1984; Rodgers et al., 1986; Trzeciak et al., 1986; Waterman et al., 1986;) such as the inner mitochondrial membrane of the corpus luteum (Rodgers et al., 1986; Rodgers et al., 1988), in bovine ovarian follicles (Rodgers et al., 1986), and in granulosa cells of developing follicles (Funkenstein et al., 1984). These systems are located in the microsomes or mitochondria of cells, depending on the type of reactions they catalyze.

Hepatic cytochrome P-450, also referred to as mixed function oxidases (MFO's), are a complex enzyme system

involving the heme protein, P-450, which catalyzes the oxidation of a wide variety of drugs, chemical toxins, environmental pollutants, fatty acids and steroids (Kaminsky et al., 1981) and are either non-specific for substrate or have overlapping substrate specificities (Kaminsky et al., 1981).

Mixed function oxidases occur in a variety of forms called isoenzymes or isozymes, depending on the type of reactions they are involved in and these isozymes can be expressed constitutively, while others are expressed as a result of hormonal or chemical stimuli (Eisen, 1986).

Cytochrome P-450 may be classified into two separate functional groups, the first being involved in the metabolism of steroid hormones in the body, by incorporation of cholesterol into the cells of steroid synthesizing organs. One of the P-450 isozymes (P-450 SCC) catalyzes the rate - limiting step in the synthesis of progesterone, estrogen, and testosterone, and is considered an endogenous substance. These isozymes are carefully regulated by protein hormones released by the pituitary, but have the capability of induction (Waterman et al., 1986).

The second is involved with the catabolism of xenobiotics and other exogenous substances foreign to normal bodily syntheses. These isozymes can be induced in vivo, by exposing them to xenobiotics or steroids not directly produced by the body. This exposure can cause an increase in concentration of

the enzyme and/or activity. Substances such as steroid hormones synthesized by the gonads and adrenal glands are known to be catabolized by the liver in rats (Thomford and Dziuk, 1986). These compounds are converted into substances excreted by the kidney in the urine or are converted in the liver and excreted from the body in the bile (Williams, 1973). The mechanism by which these enzymes hydroxylate testosterone and progesterone in the liver is unclear at this time. Thomas et al., (1987) reported that hepatic MFO's exhibited a preference for endogenous steroid hormones over drugs and xenobiotics due to the Km values for progesterone, testosterone and estradiol 17β which have been shown to be 10 times lower than those of some drug oxidations.

There are volumes of literature available describing the mechanism of steroid biosynthesis by P-450 in the gonads and adrenal glands, but very little is known about their mode of action in the liver. It is interesting that species, sex and age influence enzyme concentrations and xenobiotic metabolism (Smith et al., 1983; Stegman et al., 1988). Interpolating data from one species to another has been a major stumbling block for researchers attempting to incorporate current knowledge into applicable fields such as animal production (Watkins et al., 1987).

Induction of P-450

Cytochrome systems can be induced which results in increased enzyme concentration and/or activity. This section will discuss how the second functional group of P-450 can be induced. Jefcoate (1986) discussed in great detail four different mechanisms which rapidly affect the activity of P-450. One mechanism is the control of substrate; by increasing the amount of substrate enzyme systems respond by increasing activity, and increased enzyme activity is paralleled by accelerated drug metabolism. (Conney and Klutch, 1963).

Most data from studies on rats report increased liver size when P-450 enzyme systems are induced (Clinton et al., 1977; Truex et al., 1977; Edes et al., 1979; Eisen, 1986). Smith (1988) found increased liver size in ewes when these enzyme systems were induced with phenobarbital. A possible explanation for this response may be that the liver releases by-products from the reactions it catalyzes. These products exhibit activity involved with inflammatory responses (Waterman et al., 1986).

Earlier research (Conney and Klutch, 1963; Omura and Sato, 1964a; Omura and Sato, 1964b; Shetty et al., 1972) involving induction of liver microsomal enzymes found phenobarbital (PB) to be a potent inducer, and research was directed toward investigating the biochemical pathways

associated with measuring the enzyme systems. Numerous studies have found increased liver MFO activity in swine, rats, and sheep treated with phenobarbital (Conney and Klutch, 1963; Kaminsky et al., 1981; Thomford and Dziuk, 1986).

Shetty et al. (1972) investigated the mode of action of PB. Sheep were injected with PB over time, then antipyrine was given. Antipyrine was known at that time to be metabolized by hepatic enzymes. Their data indicated a significant increase in antipyrine plasma clearance rate in those sheep induced with PB. They interpreted the data to mean that xenobiotics actually induced the liver P-450 system. Conney and Klutch, (1963) found that rate of metabolism of testosterone and androstene 3,17 dione in rats was faster in those treated with PB. They hypothesized that treatment with PB stimulated the activity of the enzyme systems located in the liver microsomes involved in hydroxylation of testosterone and androstene 3,17 dione. They also noted that pregnenolone 16α carbonitrile is a potent inducer of drug metabolizing enzymes (Lu et al., 1972).

Nutritional Induction of P-450

Several studies have reported that quality or quantity of protein may play a key role with induction (Thomford and Dziuk, 1986; Smith, 1988; Thomford and Dziuk, 1988). It's been documented that an amino acid deficiency or toxicity may induce MFO activity. Clinton et al. (1977) conducted an

experiment to determine the influence of dietary protein level on P-450 concentrations. Growing and adult female rats were fed either 7.5%, 15%, or 45% protein diets supplemented with methionine. An increase in P-450 concentration as percent protein fed increased was observed. However, the adult rats responded to a lesser degree than younger rats due to the age-dependent decline in activity. (Clinton et al., 1977).

Edes et al. (1979) reported sulphur containing amino acids influenced MFO activity, and feeding a diet deficient in sulphur amino acids resulted in decreased MFO activity in rats. It appears that a three-way interaction has been observed. As nutritional protein levels increase, MFO activities in the liver increase, thereby increasing degradation of drugs and other exogenous substances (Clinton et al., 1977). Level of protein appears to have more effect on MFO's than energy level (Clinton et al 1977). This is supported by Truex et al. (1977) who found rats fed protein deficient diets had reduced MFO activity and drug metabolism rates. Thomford and Dziuk (1988) fed ovariectomized ewes a high (14.8%) and low (4.7%) crude protein diet. They found that feeding the high CP diet increased P-450 concentrations by 20% within 10 days of feeding and no significant difference in microsomal protein was found between treatment groups.

Nutrition and Reproduction

Studies on effects of plane of nutrition on reproduction have been done as far back as the early 1900's, and research has produced extensive results with sheep (El-Sheikh et al., 1954).

Sheep producers in Australia have found lupin grain an excellent source of protein and energy for ruminants. Many studies have investigated the effects of feeding lupin grain on ewe reproduction. Brien et al. (1981) found that ewes grazing pasture and fed lupin had significantly lower plasma progesterone concentrations than those grazing pasture without lupin supplementation. He concluded that high plane of nutrition at mating lowers plasma progesterone concentrations and suggested this may be the cause of early embryo mortality. Coop (1966) conducted an experiment to determine the influence of different planes of nutrition on bodyweight prior to and at breeding. Ewes were allocated into three groups, (HL, MM, LH) and all were of the same body condition at the time of the study. The (HL) group was fed to gain body condition for 40 d prior to flushing, then diets were switched for them to lose body condition 3 weeks prior to breeding until the end of the breeding season. The MM group was fed to maintain body condition throughout the study, and the LH group were fed for 40 d to lose body condition until the diets were switched 3 weeks prior to breeding to gain body condition throughout the

breeding season. Ewes in the HL group had higher first service conception rates, more lambed the first 17 d of the lambing season and fewer open ewes; however, the percentage of lambs born per ewe lambing was lower than the other treatment groups. The LH group had the lowest first service conception, the longest lambing season, but the highest percentage of lambs born per ewes lambing. The MM ewes were intermediate in response to the HL and LH groups.

Coop (1966) conducted a study involving time of breeding cycle and level of nutrition, without consideration of body condition. Ewes were placed in three different nutritional planes, (HH) and fed a high plane of nutrition 3 weeks prior and during the breeding season, (HL) ewes fed a high plane of nutrition prior to breeding, then switched to low during the breeding season, and (LL) ewes fed below maintenance diet prior to and during the breeding season. The results were basically the same as the study described previously; the ewes fed a high level of nutrition before and during mating (HH) showed an increase in number of lambs born per ewe mated, more open ewes, lower first service conception rates and fewer lambs born first 17 days of lambing season. Ewes fed the low level of nutrition (LL) before and during mating had the lowest fertility, fewest lambs born, and more open ewes than (HH). Ewes fed a high plane of nutrition (HL) prior to mating and switched to low plane of nutrition at mating showed higher fertility, less open ewes, but fewer lambs born per ewe mated.

Coop (1966) concluded that ewes of higher live weight at mating have improved conception rates, a lower incidence of barrenness and respond to flushing more than thinner ewes. He also stated, "special time relationship between flushing and mating is much less important than was previously thought".

Foote et al. (1959) conducted a three year study involving the effects of feeding levels on ovulation rate and embryo survival.. His data indicated a possible "carryover" effect of the flushing response such that whatever was stimulated as a result of flushing remained stimulated into the breeding season and early pregnancy. It's been postulated that the "carryover" effect may be influencing uterine environment (Edey, 1969) or possibly the peripheral concentrations of progesterone, the hormone essential for maintenance of pregnancy (Edey, 1969; Bassett et al., 1969; Bindon, 1975; Cumming et al., 1975; Tepperman, 1981; Brien et al., 1981; Williams and Cumming, 1982; Dial and Dziuk, 1983; Parr et al., 1987; Smith 1988; Rodgers et al., 1988).

Progesterone

Progesterone, a steroid hormone, is synthesized by the corpus luteum located on the ovary and adrenal glands, and is essential for the maintenance of pregnancy. This hormone can be catabolized at a faster rate than normal due to greater MFO activity (Thomas et al., 1987). Clearance rates of

progesterone influence serum concentration which affects maintenance of pregnancy.

Bedford et al. (1972) conducted a study to determine metabolic clearance rate of progesterone in sheep and found that progesterone was removed rapidly. Little et al. (1966) determined that progesterone in ovariectomized human females and in males is rapidly removed by both hepatic and extra hepatic tissues, and found no difference between single-injection of labelled progesterone and continuous infusion. It has been determined that greater than 75% of the progesterone is removed by the liver (Bedford et al., 1972). Little (1966) also mentioned that disappearance rate of progesterone from other studies were inconclusive since they were not able to determine whether the disappearance was due to metabolism or rather the distribution of the hormone into a large volume. Bedford et al. (1972) noted that there is no evidence of a 'progesterone-conserving mechanism' in sheep but in pregnant guinea pigs. There appears to be a mechanism associated with an increased concentration of a protein with high affinity for progesterone.

Effects of Nutrition on Plasma Progesterone

Parr et al. (1987) found that overfeeding during early pregnancy reduced serum progesterone concentration thereby reducing pregnancy rate in sheep. Plasma progesterone concentrations sufficient for proper embryo implantation and

support for early pregnancy (up to 50 days) lie between 2 -3 ng/ml, afterward a steady increase occurs until parturition (Bassett et al., 1969; Bindon, 1975; Brien et al., 1981, Parr et al., 1987). Parr et al. (1982) fed ewes either 25% or 100% maintenance diets from the day of mating until d 11 or 12. Plasma progesterone concentrations were higher in ewes underfed than those fed the 100% maintenance diet. Parr et al. (1987) fed three groups of ewes diets containing either a low [25% of maintenance (M)], a medium (100% M) or a high (200% M) level of nutrients. Ewes were then either implanted or not implanted (control) with 340 mg progesterone. Ewes fed the high ration had pregnancy rates significantly lower compared to the medium and low rations. Progesterone supplementation did not affect the low and medium groups, but increased the pregnancy rate in the high ration group by 28%. Progesterone concentration was less than 1.5 ng/ml for control ewes in the high ration group which is below the threshhold to maintain pregnancy (Bassett et al., 1969; Bindon, 1975; Brien et al., 1981; Parr et al., 1987). Mean progesterone concentration increased to 2.8 ng/ml when progesterone implants were used in the H group. They postulated that a higher plane of nutrition increased blood flow to the liver and progesterone catabolism (Bedford et al., 1972).

Cumming et al. (1975) imposed three planes of nutrition from the second to the sixteenth day post mating: 25%,(1/4M); 100%(M); and 200% (2M) of maintenance. Ewes fed 1/4M ration

had elevated progesterone concentrations compared to the other groups. No explanation for the rise in the underfed ewes was given. This research was similar to Williams and Cumming (1982) who fed three groups of ewes at either 1/4 maintenance, maintenance, 2X maintenance. They found that plasma progesterone concentrations were consistently higher in 1/4M ewes than with the M or 2M ewes and they concluded that there is an inverse relationship between concentration of progesterone and nutrition in ewes. Brien et al. (1981) investigated the effects of lupin grain supplements on progesterone concentrations and early embryo mortality. They concluded that a high plane of nutrition (with lupin supplementation) at mating lowers plasma progesterone and this may be a factor in early embryonic death.

Effect of Type of Protein on Reproduction

Smith (1988) in his discussion on protein intake and flushing response indicated that differences in ruminal degradation rate of protein may influence ewe reproduction. In dairy cattle, Ferguson et al. (1988) reported that cows fed diets containing greater percentage of crude protein had lower conception rates and days open, and feeding rumen protected protein improved conception rates and decreased number of services/conception and days open. They hypothesized that feeding excess ruminally degradable protein lead to higher concentrations of ammonia, urea or other

nitrogenous compounds in the blood and uterine fluids that are toxic to spermatozoa, ova or embryos. Feeding a high crude protein ruminally degradable feed increased blood urea nitrogen (BUN) over cows fed rumen-protected protein. He concluded BUN concentrations greater than 20 mg/dl may predispose cows to infertility. Thompson et al. (1973) found that ewes fed purified diets containing urea as the major N source required several services before conception occurred. However, in their study they found no detrimental influence on reproductive efficiency due to feeding urea to ewes, cows, rams, and bulls.

Several authors (Menard and Purvis, 1973; Thomas et al., 1986; Smith, 1988; Thomford and Dziuk, 1988) reported that excess protein or merely overfeeding influences enzymatic activity which in turn affects hormonal levels associated with early embryo mortality.

Menard and Purvis (1973) indicated that enhanced production of steroids stimulated P-450 concentration in the liver. He postulated that due to the increase in microsomal enzymes, estradiol metabolism increased, thereby causing more FSH circulation, and increased ovulation rates. Thomas et al. (1987) made the same inference. When mixed function oxidase activity is increased, steroid hormones are catabolized at a faster rate, which in turn influences a greater negative feedback on the pituitary and hypothalmus (Thomford and Dziuk, 1988). Smith (1988) mentions that in looking at the mechanism

underlying the flushing response, estradiol 17β depresses ovulation rate in the ewe and that it appears to lower Follicle Stimulating Hormone (FSH) levels as well. FSH is a protein hormone released by the pituitary gland and promotes follicular growth and development in the ovaries. Therefore, when removed from peripheral blood at a faster rate due to a higher MFO activity in the liver, estradiol 17β will reduce the negative feedback control on the hypothalmus and pituitary, thereby increasing LH and FSH release from the pituitary, and thereby causing more follicles on the ovary to develop (Thomas et al., 1987).

In summary, nutrition can affect reproduction by influencing enzymatic activity in the liver. Flushing and associated high plane of nutrition has a three week carryover effect on reproduction. Since flushing is practiced before and during the breeding season, this "carryover effect" may be of major importance to early pregnant ewes. It has been shown that overfeeding induces liver MFO activity which in turn catabolizes progesterone at a faster rate. Thus early pregnant ewes are subject to losing embryos from their first service due to reduced progesterone concentrations which is insufficient to maintain pregnancy.

The purpose of this study is to determine what type of excess protein fed will stimulate MFO activity and to determine whether progesterone disappearance rate increases as MFO activity increases.

CHAPTER 3

MATERIALS AND METHODS

Animals

The experiment was conducted at the Fort Ellis Agricultural Experiment Station near Bozeman, Montana.

Rambouillet ewes from three genetic lines developed by Burfening et al (1989) were used. Ewes were either selected for high (H) or low (L) reproduction based on their dam's reproductive rate beginning in 1969. By 1972, the remaining foundation ewes from L and H were random bred to form the control (C) line.

Nineteen Rambouillet ewes were available from a previous study which consisted of H line (n=4), L line (n=9) and C line (n=6). These ewes had undergone ovo-hysterectomies. The use of ovariectomized ewes should minimize changes in steroids due to estrus cycles which could affect mixed function oxidase levels (Thomford and Dziuk, 1988).

The trial began June 1, 1989 when liver biopsies were taken to determine baseline values for P-450 and a repeat biopsy was taken on June 27, 1989. Following the second biopsy ewes were fed their experimental diets until July 11, 1989 when a 12 h window bleed for progesterone was conducted prior to the study the ewes were maintained on pasture.

Facilities

Ewes were penned individually in 1.46 m^2 pens. Ewes were in close proximity of each other and bedded with wood chips. They were protected from inclement weather and wind to minimize stress. Ewes were fed twice daily and clean water was available at all times. Surgeries were performed in the surgical facilities located at the Fort Ellis Agricultural Experiment Station.

Treatments

Following the initial liver biopsies, nineteen ewes were randomly assigned to one of two dietary protein supplement groups: blood meal (BM) or urea (U). Pelleted diets were formulated to be isocaloric and isonitrogenous (Table 1). Quantity fed was calculated at 1.5 times maintenance protein requirement for non-pregnant dry ewes (NRC, 1985). This level of dietary protein was used to ensure a stimulation of P-450 concentration (Thomford and Dziuk, 1988).

Diets were fed twice daily at approximately 0830 and 1700 h. Ewe weights were recorded prior to and at the end of the trial.

Surgical Procedure

On June 1, 1989, liver biopsies were taken surgically from 18 ewes. One ewe reacted to the biotal and was removed from the project. Repeat biopsies were taken on June 27, 1989. Food and water were withheld twelve hours prior to the

Table 1. INGREDIENT AND NUTRIENT COMPOSITION OF EXPERIMENTAL DIETS

	Protein Supplement	
Ingredient (%)	UREA	BLOOD MEAL
Straw	81.1	83.9
Molasses	5.9	7.8
Wheat Millrun	8.0	
Urea	.9	
Blood Meal		4.0
Gypsum	.3	.2
Dicalcium phosphate	.1	.5
Bentonite	3.2	3.2
Salt-Iodized	.2	.2
Trace mineralized salt	.2	.2
Dry matter and nutrient conc.		
Dry matter intake, kg	1.86	1.82
Crude protein, %	7.70	7.30
Metabolizable energy, Mcal kg^{-1}	3.73	3.98

surgeries. Liver biopsy samples were obtained from eighteen and sixteen ewes respectively during the initial and repeat surgery.

Ewes were shaved on the right side, from the ninth rib to 10 cm caudal to the end of the last rib. Twelve ml of 4% Biotal (Bioceutics) were given I.V. in the carotid artery with an 18 x 1-1/2 inch needle. When the ewe became prone, she was placed on a surgery table, and halothane immediately

administered. Lying on her left side, the shaved area was surgically scrubbed and a sterile surgery drape was used. An incision was made starting approximately 5 to 10 cm caudal and 5 to 10 cm lateral to the sternum and then continuing for 10 cm parallel to the end of the ribs with an electro-surgical unit. Both muscle layers and peritoneum were opened, and a "thumbnail" liver biopsy was taken by sliding two fingers and thumb into the incision moving toward the ribs and pinching off 1 to 5 g liver. Wisniewski et al. (1986) found uniformity of hepatic xenobiotic metabolizing enzymes throughout the livers of cattle, goats and sheep. Therefore, we were not concerned about sampling from the same lobe each time. Liver samples were immediately placed on ice and kept at 0° C until microsomal preparations were performed. Peritoneum and each muscle layer, were sutured and the skin surgically clamped. Twelve ml of penicillin were administered intramuscularly. Each ewe was then placed in an indoor pen adjacent to the surgery room and kept under surveillance for 24 h. Hay and water were available. Following the twenty-four hour surveillance period, ewes were placed back in their pens and fed their respective diets.

Microsomal Preparation and P-450 Analysis

Liver samples were taken, weighed and recorded, and rinsed in cold .15M KCL. Each sample was placed in a glass tube marked with the ewe's identification number, and stored

on ice. Liver enzyme assays were completed on the same day of the surgeries in a cold room kept at 0-4° C.

Two to five g of sample were minced with scissors, and rinsed three times in approximately 10 v .15M cold KCL to remove all blood. Minced samples were then placed in a 15 ml teflon-glass homogenizer and 10% w/v of Tris Buffer (.05M Trizma/1.5M KCL, pH 7.4) was added. Samples were homogenized for approximately 60 seconds, and centrifuged at 12,000 x g for 20 minutes at 4° C.

Centrifuge tubes were weighed, marked and recorded. After the centrifugation was completed, supernatant was poured off into the ultra-centrifuge tubes. Supernatant was centrifuged at 100,000 x g for 90 minutes to obtain a microsomal pellet. After completion of the last spin, the supernatant was poured off and a reddish-brown gel-like pellet was obtained. Pellet weight was recorded.

Pellets were resuspended to approximately 20% w/v with Tris-Buffer and microsomal protein analysis conducted using Pierce BCA protein assay reagent[a]. Resuspended pellets were diluted to approximately 5 mg protein ml^{-1} A baseline was recorded on the sample, then the sample was reduced with sodium dithionite, and another baseline recorded. Carbon monoxide was bubbled in, and difference spectra was recorded from 400-500 nm.

[a]Pierce Chemical Company, 3747 Meridian Road, Rockford, IL 61105

An extinction coefficient of 91 nM^{-1} cm^{-1} was used to calculate cytochrome P-450 concentration.

Progesterone

Five ewes from each protein supplement group were randomly selected for use in a window bleed. On July 11, 1989 indwelling jugular catheters were placed in each of the ten ewes. One ewe pulled her catheter out shortly after insertion and was removed from the window bleed. A 10 ml blood sample was taken at 0930 for baseline progesterone and blood urea nitrogen (BUN) analyses. At 0945, 25 mg progesterone was injected through the catheters followed by 5 ml saline. At 1000 the first blood sample was taken, in increments of 15 min for the first 2 h, then every 30 min for the next 2 h, then every 60 min for the remaining 8 h. Blood samples were refrigerated immediately until centrifugation. Blood samples were centrifuged for 30 min at 12,000 x g. Upon completion of centrifugation, serum was poured into serum tubes and frozen at -20° C.

Serum samples were sent to Dr. Dennis Hallford, Endocrine Laboratory at New Mexico State University, Las Cruces, New Mexico for progesterone analyses. Validation for the assay was done according to Hallford et al. (1982). The Progesterone - 11 BSA antiserum (GDN 337) was kindly supplied by Dr. G.D. Niswender, Department of Physiology and Biophysics, Colorado State University, Fort Collins, Colorado. Serum samples were

analyzed for BUN using an Ames Pacer semi-automated analyzer[b].

Statistical Analysis

Microsomal Protein, P-450, BUN and weight data were analyzed using a completely randomized design with least-squares analysis of variance using the General Linear Models Procedure of SAS (1987). The statistical model included the fixed effects of protein supplement (BM vs U), Line (H,L,C) and their two-way interaction. Means were separated using least significant difference. Progesterone data were analyzed SAS (1987). Model included the fixed effects of treatment and by least squares analysis of variance by the GLM procedure of linear and inverse quadratic regressions of time. Regressions were initially evaluated within time to determine differences in disappearance pattern. Regression values were not different (P>.10) and therefore regression values within treatment were combined. Linear regression was not different (P>.10) from zero.

[b]Miles Laboratory, Inc., Elkhart, IN.

CHAPTER 4

RESULTS AND DISCUSSION

Initial and final live weights were not different (P>.10) between protein supplement groups (Table 2). Mean initial and

Table 2. INFLUENCE OF PROTEIN SUPPLEMENT ON EWE BODY WEIGHT AND CHANGE, BLOOD UREA NITROGEN AND MICROSOMAL PROTEIN CONCENTRATION

	Protein Supplement			
	BM	U	SE[a]	P
Weight, kg				
Initial	60.7	59.7	5.68	0.78
Final	61.7	61.0	6.24	0.84
Change	.99	1.19	1.19	0.79
Blood Urea N, mg dl^{-1}	8.2	13.2	1.87	0.12
Microsomal Protein, (mg g^{-1})				
Pre-treatment	18.12	17.82	0.96	0.83
Post-treatment	13.51	14.58	0.49	0.14
Change	-4.74	-3.15	1.32	0.45

[a]Standard error of least square means

final weights were 60.7 and 61.7 kg for BM and 59.7 and 60.9 kg for U, respectively. Weight change during the trial for BM and U were similar (P>.10); .99 and 1.19 kg, respectively.

Microsomal protein concentrations pre and post-treatment for BM and U were 18.12 and 17.82 mg g^{-1} liver (pre) and 13.51 and 14.58 mg g^{-1} liver (post), respectively, with no difference (P>.10) between treatments (Table 2). Pre and post treatment microsomal protein concentrations lie in the range found by Thomas et al. (1987). No differences (P>.40) in change in microsomal protein concentrations were detected. Associated enzymes and P-450 involved with hydroxylation of drugs are found in the microsomes of the liver. It has been shown that phenobarbital and other xenobiotics have the ability to induce the formation of microsomal enzymes and P-450. (Mayes, 1988). Our data are in agreement with that of Thomford and Dziuk (1988) who reported that increased protein intake had no effect on microsomal protein concentrations on liver of ewes but did elevate cytochrome P-450 concentrations. Average post-treatment concentration of microsomal protein in our study (14.08 mg g^{-1}) was higher than their reported values (7.7 mg g^{-1}).

Calculated pre and post-enzyme concentrations for individual ewes are reported in Table 3. Range of values for enzyme concentrations agree with other published values (Thomford and Dziuk, 1986; Thomas et al., 1987; Wisniewski et al., 1987; Thomford and Dziuk, 1988).

Pre-treatment enzyme concentrations were not different (P>.10; .69 BM vs .92 U nmol mg^{-1} protein). However, post-enzyme concentrations were different (P<.05) with U fed ewes

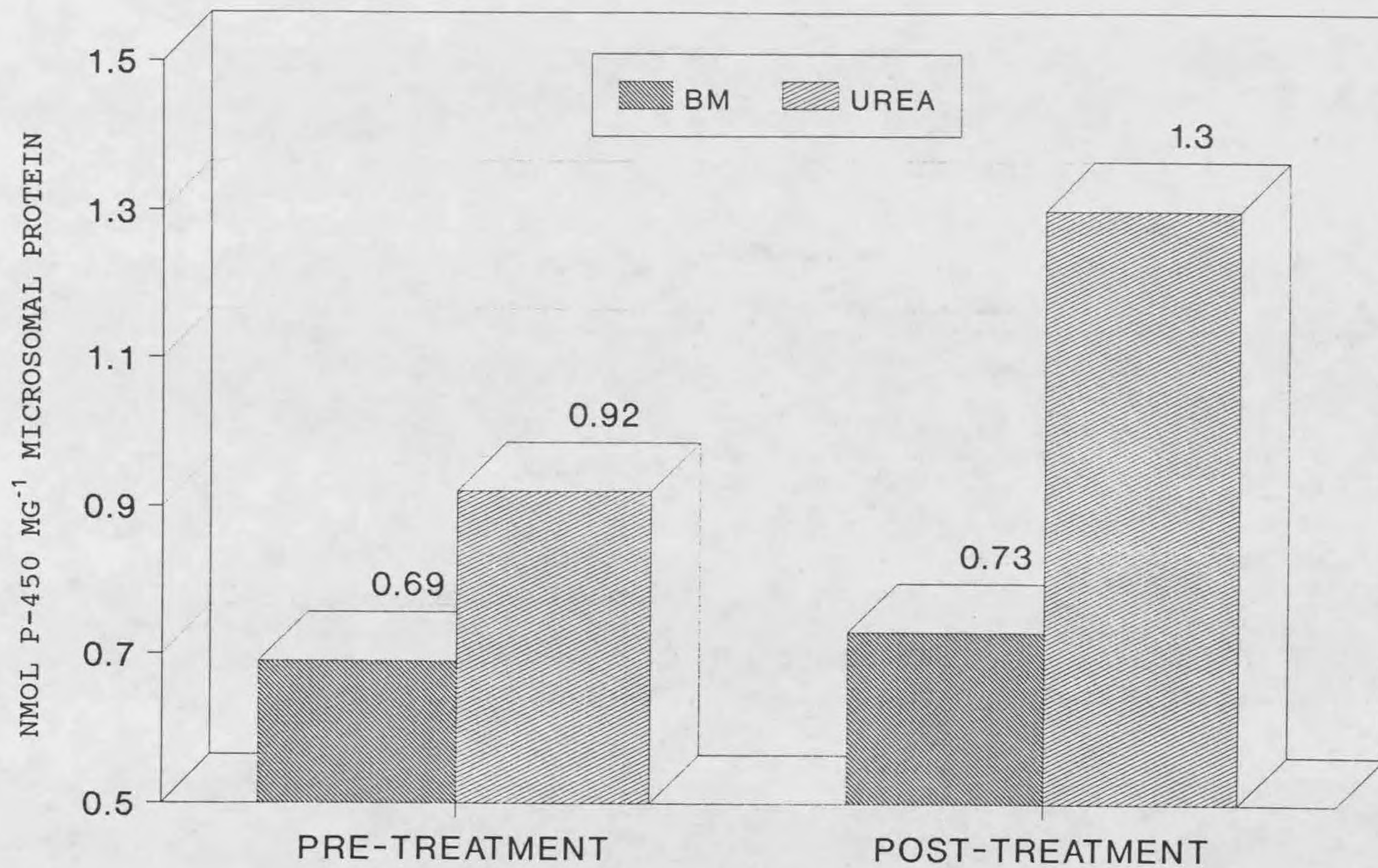
Table 3. P-450 CONCENTRATIONS PRE AND POST-TREATMENT BY EWE[a]

Ewe ID	Protein Supplement	P-450 Concentration Pre-treatment	Post-treatment
2002	BM	.9080902	1.3182560
2008	BM	.3389306	.9478077
2011	BM	.9313917	--------
2017	BM	.8713542	.5030785
2019	UREA	.7764586	.6662652
2028	BM	.5347362	.5347362
2029	UREA	1.0720560	.7760003
2048	BM	.8038958	1.0273220
2054	UREA	.4947130	--------
2422	BM	.6138049	.7697201
2429	UREA	1.0141120	2.0480870
2473	UREA	.7211695	1.7380780
2645	UREA	1.0317550	1.0695950
2648	UREA	1.1571450	1.6214110
2650	BM	.7709571	.6323807
2655	UREA	.7148759	1.0664330
2679	UREA	.9354623	1.3469060
2705	BM	--------	.8995252

[a]P-450 concentrations in nmol mg^{-1} microsomal protein

having greater concentrations of cytochrome P-450 than the BM group (Figure 1; 1.3 vs .73 nmol mg^{-1} protein). These data suggest a relationship between dietary protein source and cytochrome P-450 concentration. Dry matter and protein intakes between treatment groups were similar (128.8 g^{-1}, BM versus 138.4 g d^{-1} protein, U). Therefore, differences in cytochrome

FIGURE 1. Least Square Means of P-450 as Influenced by Protein Supplement



P-450 should be related to protein source and not protein intake. Ewes fed U tended (P>.10) to have higher concentrations of BUN (Table 2) than those fed BM with 8.2 mg dl^{-1} in the BM group and 13.2 mg dl^{-1} for U. BUN values are indicative of N catabolism in the body. In our situation, excess N was probably available from urea and converted to ammonia in the rumen. Ammonia was absorbed into the portal blood system and was filtered in the liver. Thus the liver may have increased cytochrome P-450 activity to clear excess ammonia to prevent ammonia buildup in the body (Conney and Klutch, 1963; Jefcoate, 1986; Rodwell, 1988). Change in P-450 concentration (Figure 2) therefore tended (P=.15) to be greater for those fed U compared to BM (.04 vs .38 nmol mg^{-1}).

Pre and post-treatment cytochrome P-450 concentrations were not different (P>.10) between lines (Figure 3). Pre-treatment least square means of enzyme concentration in nmol mg^{-1} microsomal protein for H, L, and C lines were .74, .87, .81, respectively. Although not significantly different (P>.10) H line ewes tended to have greater P-450 concentrations than the other lines. This resulted in a significant change (P<.10) in P-450 concentration in H line compared to C or L line ewes (Figure 4).

It has been documented that the H ewes are superior to the L ewes in litter size, but were significantly lower in first service conception (P<.05) although no fertility differences were found over all services (Schoenian 1988).

FIGURE 2. Least Square Means of Change in P-450 as Influenced by Protein

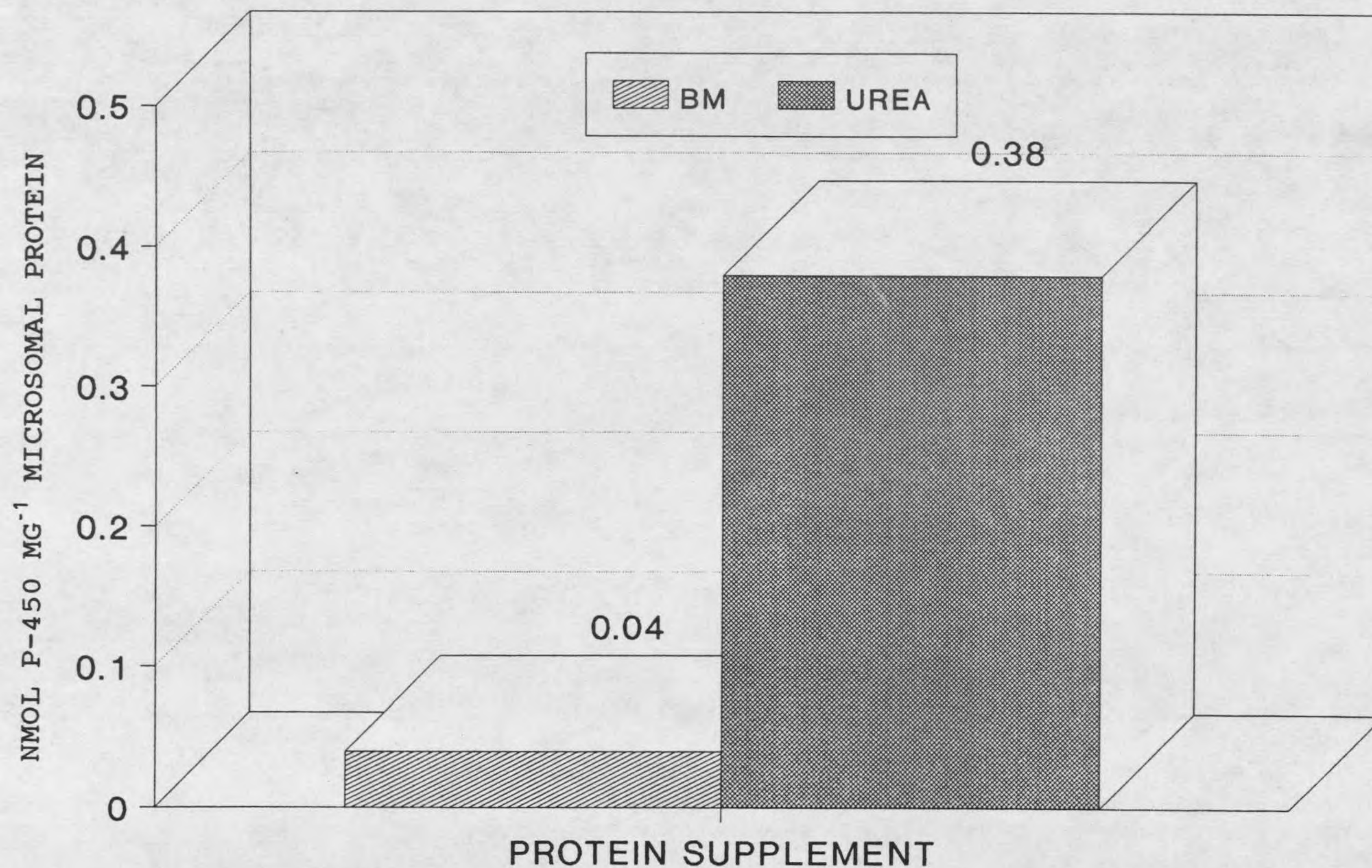


FIGURE 3. Least Square Means of P-450 as Influenced by Line

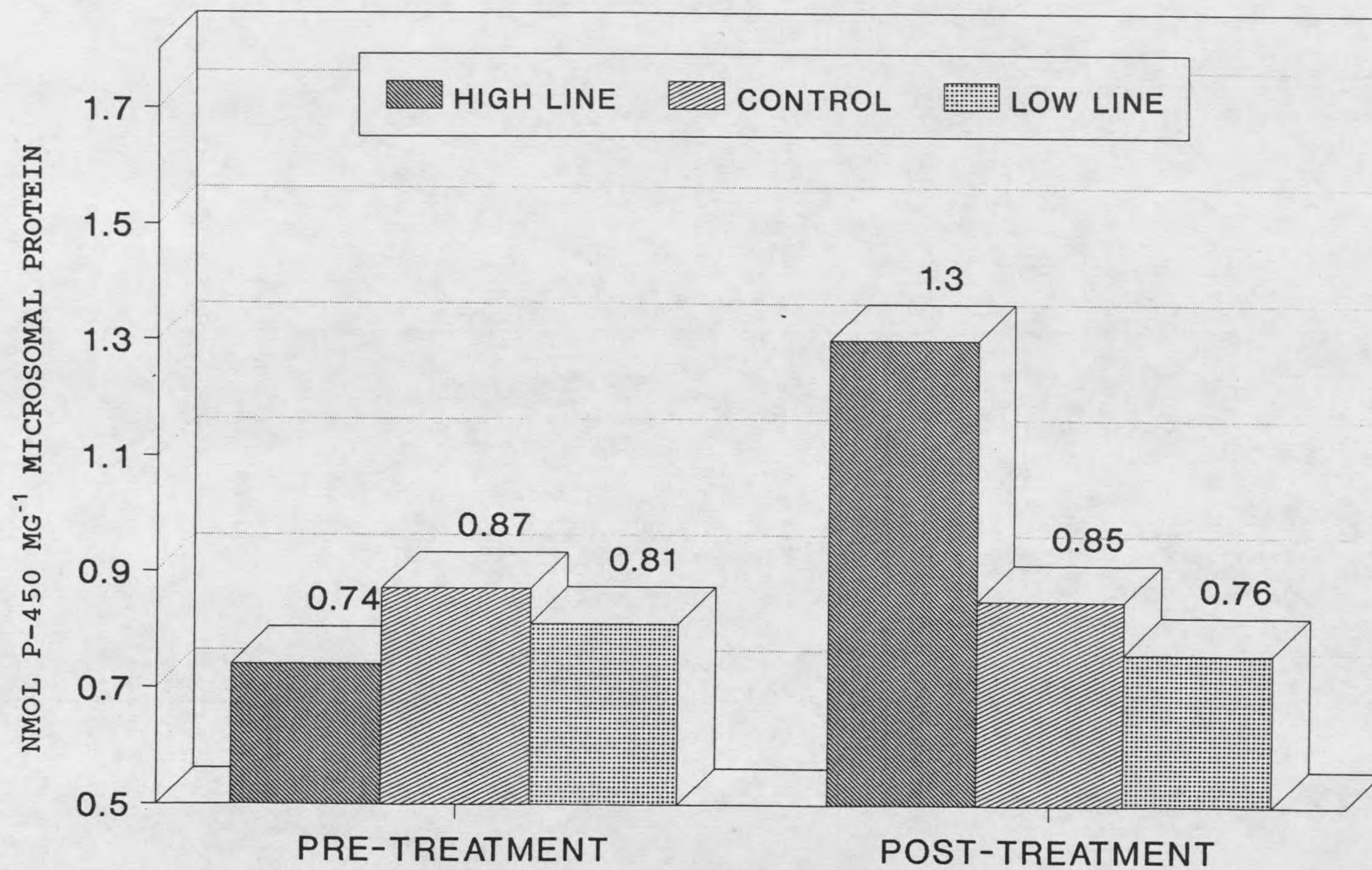
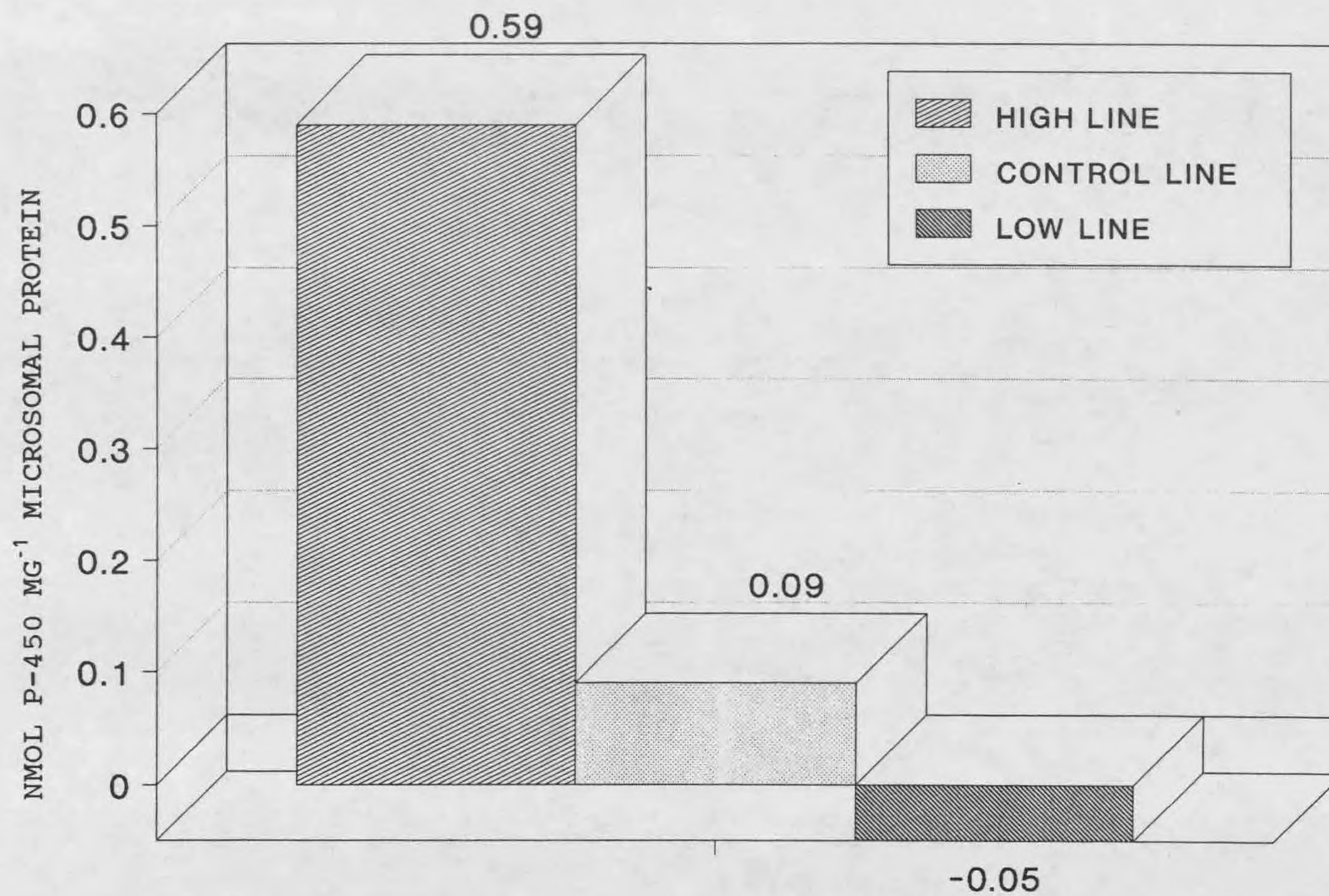


FIGURE 4. Least Square Means of Change in P-450 as Influenced by Line



In Schoenian's master's thesis she stated, "Embryo survival was lowest in the H line". We suggest lower progesterone concentrations may be the underlying cause of this due to the increased mixed funtion oxidase activity in the liver catabolizing progesterone at a faster rate (Menard and Purvis, 1973; Thomas et al., 1986; Smith, 1988; Thomford and Dziuk, 1988). Although it was not the primary objective of our work to determine differences in P-450 concentrations between reproductive lines, these data suggest selection for reproduction may be influencing liver enzyme systems.

A significant treatment by line interaction was detected (P<.05) with respect to P-450 concentration and change. (H) line ewes fed U had greater (P<.05) concentrations of P-450 than all other treatment combinations (Figure 5). High line ewes fed BM and U had values of .77 and 1.9 respectively, (C) BM and U .63 and 1.3, respectively and (L) were .8 and .72 nmol/mg microsomal protein respectively. Therefore, P-450 concentration change was greatest (P<.05) in the (H) line fed U (Figure 6).

However, these results must be treated with caution since the number of ewes from H line was small, (n = 3) with 2 H line ewes in the U group and 1 in the BM group.

Progesterone Disappearance Rates

Quadratic regression was significant (P<.01; Table 4) for best fit of the data points. No difference (P>.10), however

FIGURE 5. Least Square Means of Post P-450 as Influenced by Protein and Line

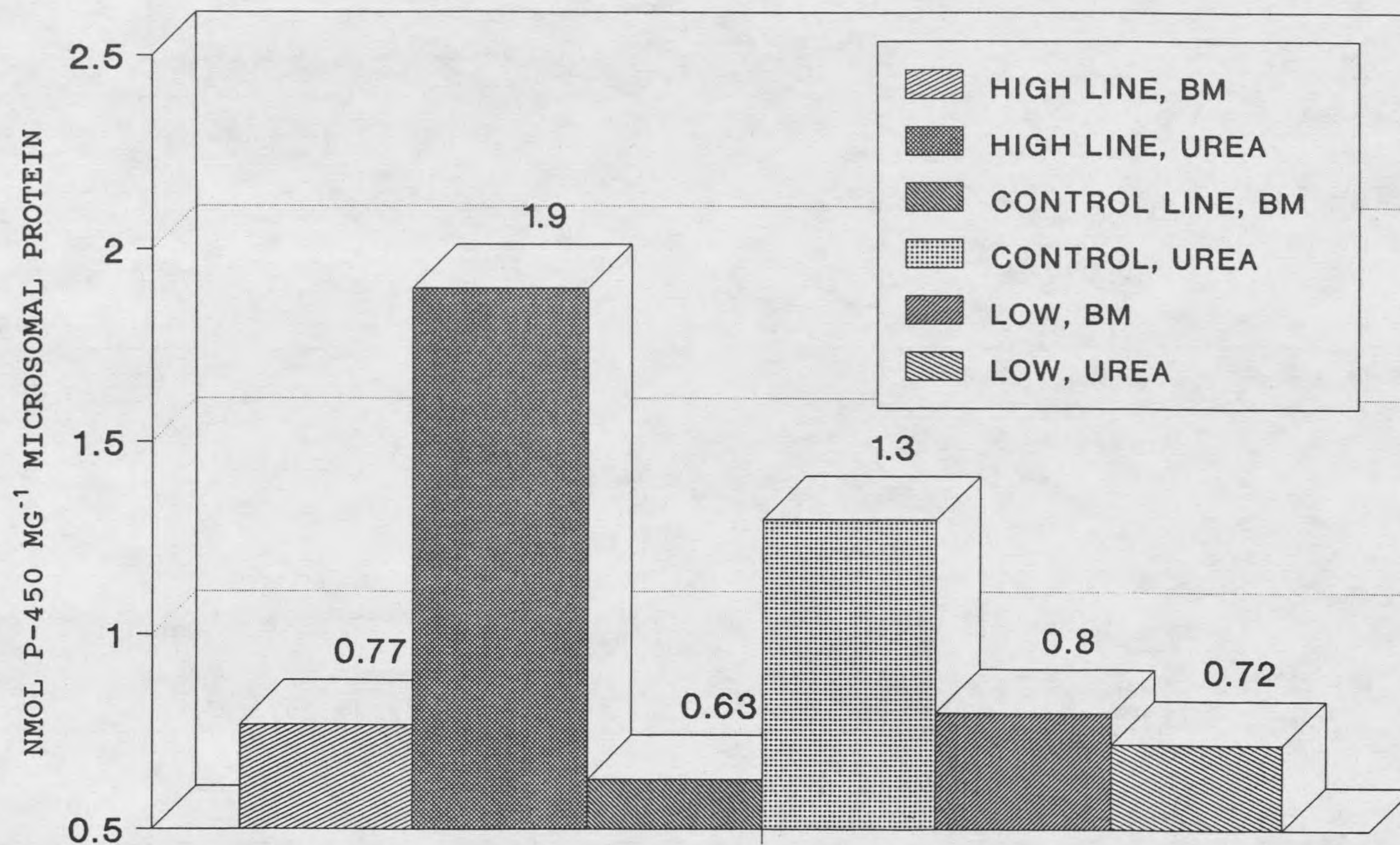


FIGURE 6. Least Square Means of Change in P-450 as Influenced by Protein and Line

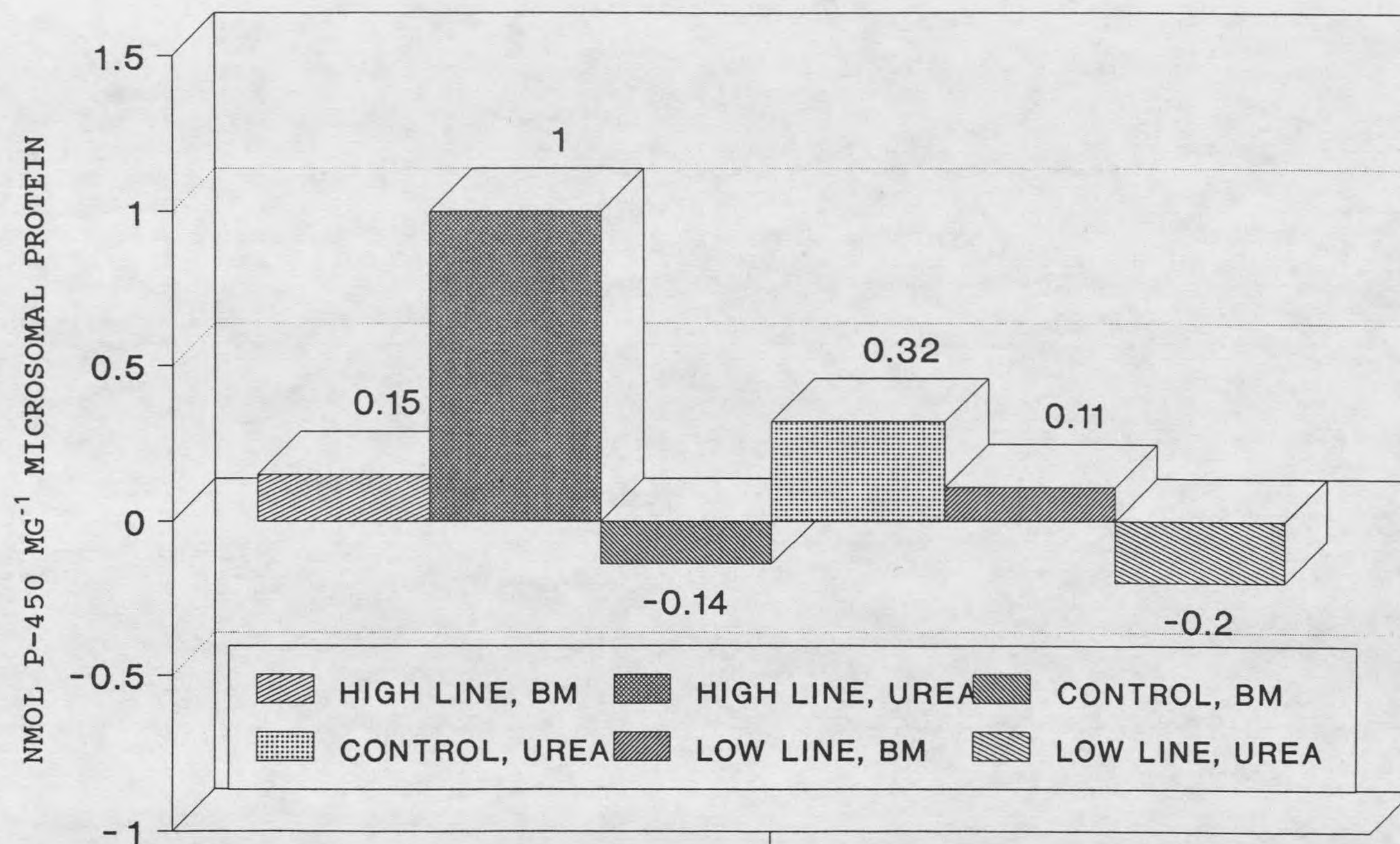


Table 4. ANALYSIS OF VARIANCE FOR LINEAR AND QUADRATIC REGRESSION ON PROGESTERONE[a]

Source	DF	Mean Square	F Value	P
Treatment	1	2.2	0.90	0.34
Ewe (Trt)	5	11.2	4.56	0.0007
Linear	1	0.5	0.21	0.65
Quadratic	2	1070.2	433.5	0.0001

[a] R^2=.88; Ewe (Trt) used to test treatment.

was detected between protein supplement treatments. Least square treatment means were: U, 1.69±.20 and BM 2.02±.17. It is apparent when looking at progesterone concentrations in Table 5 that time had more effect on progesterone disappearance than did treatment. Treatment means and standard deviations within time were similar. It appears from the data that more frequent sampling should have occurred immediately following progesterone injection, progesterone could have been infused in the side opposite the sampling site, and perhaps 1 mg progesterone could have been infused.

Table 5. MEAN PROGESTERONE CONCENTRATIONS UG/ML BY TIME WITH STANDARD DEVIATIONS

	TREATMENT			
TIME (min)	BM	STD DEV	UREA	STD DEV
0	19.12	6.93	20.66	8.7
15	4.11	2.01	2.80	0.94
30	2.20	0.90	2.92	1.27
45	1.27	0.58	1.51	0.29
60	1.13	0.26	1.41	0.26
75	0.91	0.28	1.08	0.25
90	0.91	0.20	1.28	0.64
>90	0.42	0.10	0.77	0.56

CHAPTER 5

CONCLUSION

Progesterone disappearance rates were probably not meaningful since progesterone injection and sampling sites were the same, and blood samples were probably not taken frequently enough (recommend 5 incremental blood samples for first 30 minutes, followed by 15 minute samples drawn for next 3 h). This study demonstrated that protein quality influenced cytochrome P-450 concentrations. The inducer of the cytochrome system in our experiment appeared to be ruminal ammonial concentrations and not amino acids. It was interesting that the cytochrome P-450 system seemed to be more induced in ewes fed U that had been selected for high reproductive rate. This information presents a number of questions involving the relationship to genetic selection and response to environment. Perhaps genetics is simply selecting for more enzymes, or their sensitivity to the environment and/or substrate. A few questions I pose for further research are; Have the H line ewes been selected for synthesizing more of these enzymes? Are their enzyme systems more sensitive to different substrate? Have the L line ewes been selected for little response to different substrates?

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