To the Graduate Council:

I am submitting herewith a dissertation written by Sou Fei Chin entitled "Relation of Arginine Nutrition to Mammary Gland Development." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

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RELATION OF ARGININE NUTRITION TO MAMMARY GLAND DEVELOPMENT

A Dissertation

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Degree

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Sou Fei Chin

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ABSTRACT

Experiments were conducted to determine the effect of dietary arginine on mammary gland development in gravid and nongravid rats. Dietary arginine ranged from 0% to 0.75%. All rats were weighed and euthanized within 8 h after parturition. Both mature and nongravid rats fed arginine-free diets remained in positive nitrogen balance but lost body weight. However, as little as 0.15% dietary arginine increased nitrogen retention nearly 3-fold and prevented weight loss. Number of pups born and their birth weight were not affected by dietary arginine. Consumption of an arginine-free diet during gestation significantly reduced mammary gland weight and mammary gland weight as a percent of body weight. Depression in mammary gland weight was associated with depressed significantly mammary RNA and DNA concentrations. However, the RNA:DNA ratio was not affected by dietary arginine. Increased mammary mass and nucleic acid content was not attributed to increased feed intake, since feed intake of gestating rats increased only 35% while arginine intake increased by 300%. Restricting feed intake by pair-feeding resulted in body weight loss; however, mammary gland mass and nucleic acid content were significantly higher than that of rats fed an arginine free diet. Arginase and S-adenosyl-L-decarboxylase activities were not affected by dietary arginine concentration.

Arginine level significantly affected ornithine decarboxylase activity, but the effect was inconsistent. There was a high correlation between ornithine decarboxylase activity and RNA concentration. Restricting arginine or feed intake during early gestation followed by excess intake of arginine or feed resulted in compensatory growth of rat mammary glands. Arginine requirement for nongravid and gravid rats based upon nitrogen retention and body weight gain was estimated to be 0.21% and 0.55%, respectively. This study demonstrated that dietary arginine is required for optimal mammary gland development during gestation. It appears that the effect of arginine is not mediated directly via ornithine decarboxylase.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER I

INTRODUCTION

Polyamines are naturally occurring compounds related intimately to cell differentiation, macromolecule synthesis and growth (Tabor and Tabor, 1984; Pegg, 1986). Key enzymes in the biosynthesis of polyamines are ornithine decarboxylase and S-adenosyl-L-methionine decarboxylase. The activities of these two enzymes is relatively low in nonproliferating tissues and is induced by a wide variety of stimuli such as hormones, growth factors, tumorigenic viruses and dietary protein (Pegg, 1986).

Pregnancy in mammals induces substantial morphological and functional modifications of the mammary gland. Growth of the mammary gland is slow during the first half of pregnancy, but increases as pregnancy advances (Hollmann, 1974). The increase in mammary gland DNA synthesis during pregnancy is about 55-75% in the rat (Munford, 1963b). Tucker and Reece (1963a) reported that early in gestation RNA concentration increased in parallel to DNA concentration. However, as gestation progressed to the later stage, DNA concentration plateaud and RNA concentration continued to increase. The concentration of two polyamines, putrescine and spermidine, in rat mammary glands remained low in the virgin state, and rose during pregnancy as proliferation of mammary epithelial cells occured (Russell and McVicker, 1972). The increase in

concentrations of putrescine and spermidine were accompanied by increased in the concentrations and activities of arginase, ornithine decarboxylase, S-adenosyl-L-methionine decarboxylase and spermidine synthase (Russell and McVicker, 1972), which indicates that polyamines may be involved in proliferation of mammary epithelial cells.

Tabor and Tabor (1984) suggested that arginine may serve as a precursor of polyamines which are involved in mammary growth and milk synthesis. Formation of polyamines from arginine was shown by polyamine-requiring mutants of Chinese hamster ovary cells (Pohjanpelto et al., 1981), in which defective arginase adversely inhibited de novo synthesis of putrescine. Injection of arginine into rats produced very marked increases in ornithine decarboxylase activity (Fausto, 1970). Hence, it appears that arginine intake may affect ornithine decarboxylase activity and polyamine synthesis which in turn may affect mammary gland growth.

Arginine is considered a nonessential amino acid in mature humans (Rose et al., 1954), pigs (Baker et al., 1966), and rats (Burroughs et al., 1940). Recent studies provided evidence that feeding arginine-deficient diets decreased nursing performance, mammary gland weight, and total DNA and RNA contents of gravid rats; but no change in the RNA:DNA ratio was observed (Pau and Milner, 1981, 1982). These studies suggest that feeding an arginine-free diet to gravid rats markedly impairs mammary gland development by decreasing mammary gland mass and nucleic acid content.

However, the concentration of dietary arginine that is required for optimal reproductive performance has not been investigated, and the role of arginine in mammary gland development is still unclear.

Objectives of experiments reported herein were to study the requirement for, and effect of, dietary arginine during gestation in the rat; and to examine possible relationships of dietary arginine and polyamine biosynthesis with mammary gland development.

CHAPTER II

LITERATURE REVIEW

Mammary gland development occurs during pregnancy, and continues during the early part of lactation (Cowie et al., 1980). In pigs, the fastest rate of mammary growth, as indicated by DNA concentration, occurs between days 75 and 90 of pregnancy (Kensinger et al., 1982). Several key hormones and diet regulate mammogenesis and lactogenesis. Mammogenesis involves all cell functions which lead to an increase in total epithelial cells. Lactogenesis involves synthesis, within mammary epithelial cells, by the necessary machinery to produce milk and to release it into the lumen of the alveoli (Ceriani, 1974).

MORPHOLOGICAL AND PHYSIOLOGICAL CHANGES IN MAMMARY GLAND DURING GESTATION

During pregnancy, mammary parenchyma is transformed into a fully secreting tissue. This process requires growth of the glandular tree and differentiation of resting gland cells into functional cells. The sequence of events is quite similar in different species, and only their time course varies with length of pregnancy (Hollmann, 1974). Growth of the mammary gland is slow during the first half of pregnancy, but the rate of growth increases as pregnancy

advances. In the mammary gland of the virgin animal, a fairly prominent fatty pad exits. Fat pad adipose cells are replaced gradually with ducts, lobule alveoli, blood vessels, lymph vessels and connective tissue structures of collagen and elastin (Anderson, 1985). In mice, the first lobules are formed at eight to ten days of pregnancy. Alveoli become rounder, glandular cells become columnar, and their infolded nuclei occupy a great portion of the cell. The cytoplasm is more abundant and contains numerous mitochondria and Golgi vacuoles. A few protein granules enclosed in vacuoles and lipid droplets of various sizes are scattered throughout the cytoplasm and occupy about 20% of the cytoplasmic volume (Hollmann, 1974). Munford (1963a) showed that the volume of glandular tissue increases steadily from early pregnancy to a maximum value in the middle or latter part of pregnancy in the rat.

During the second half of pregnancy, growth of the gland continues and secretory lobulo-alveolar activity increases slowly (Hollmann, 1974). Lobulo-alveolar development becomes complete towards parturition, but the glandular lumina remains narrow. At the second or third day antepartum, a marked increase in the amount of rough endoplasmic reticulum occurs. Alveolar cells contain an abundance of small lipid droplets and Golgi vacuoles containing many protein granules with little clear fluid (Hollmann, 1974). Munford (1963a) reported that in the rat and to a lesser degree in the mouse, there was a marked fall

in the number of alveoli per unit area at the time of parturition and a corresponding increase in alveolar size. Chatterton et al. (1975) reported that in the last hours before parturition, the secretory products, protein and lipid, are extruded rapidly and enter the alveoli cavities.

Increase in cell size, cellularity, area, and DNA concentration during pregnancy in different species have been reported by several researchers. Foster (1977) used cell volume to estimate developmental changes in mouse mammary glands during pregnancy and lactation. On day 4 of pregnancy, a significant increase in cell volume over that of tissue from virgin mice was observed, but the major enlargement in cell volume occurred between day 16 of gestation and parturition, followed by an additional increase during lactation. Aveolar cell size was relatively constant during the latter part of pregnancy and in lactation, but increased two-fold at the time of parturition in the rat (Munford, 1963a). Increased mammary gland DNA synthesis during pregnancy was about 55-75% in the rat and 61% in the mouse (Munford, 1963b). Mammary gland DNA concentration was doubled by day 14 of pregnancy, and at parturition, was seven times greater than in nonpregnant rats. About 30% of the total growth of the mammary gland during pregnancy and lactation in mice took place between days 6 and 12 of gestation and about 47% occurred between day 12 and parturition (Brookreson and Turner, 1959). The remaining 20% of mammary growth took place during the first

14 days of lactation. Total RNA content of the mammary gland in mice increased steadily throughout pregnancy, and the RNA:DNA ratio was less than one until just before parturition (Yanai and Nagasawa, 1971). Tucker and Reece (1963a) reported that the total DNA content of the mammary gland increased throughout pregnancy and continued until day 10 or 12 of lactation.

Only about 60% of total mammary growth in the rat takes place during pregnancy (Griffith and Turner, 1961). Early in gestation, RNA concentration increased in parallel to DNA concentration. However, as gestation progressed to the later stage, the rate of RNA increase surpassed rate of DNA. Thus, there is a gradual increase in RNA:DNA ratio (Tucker and Reece, 1963a). A further increase in RNA content occurs during the first 14 days of lactation and the RNA:DNA ratio triples during this period (Tucker and Reece, 1963b). Paape and Sinha (1971) demonstrated that the concentration of DNA continued to increase into early lactation in the rat.

HORMONAL REGULATION OF MAMMARY GLAND DEVELOPMENT AND FUNCTION

During very early fetal development, the rudimentary mammary gland apparently is free of hormonal control (DeHoff et al., 1986), however, subsequent mammary development is dependent upon hormonal stimulation. Hormones required for proper mammary gland development include general metabolic

regulators such as insulin, thyroid hormone and corticoids, as well as specific stimulatory hormones such as estrogen, progesterone, prolactin and growth hormone (Anderson, 1974). Many studies have shown that ovarian steroids are necessary to induce lobuloalveolar formation in mammary tissue. Two prominent effects of estrogen are (1) stimulation of ductal growth and (2) an increase in progesterone receptor concentration (Haslam, 1987). Yoshinaga et al. (1969) observed that during the first week of pregnancy in rats, estrogen secretion increased on the third and fourth days, followed by a period in which plasma concentrations were negligible. From days 14 to 15, the secretion of estrogen increased, reaching very high levels between the eighteenth day and parturition. Plasma estrogen concentration disappeared entirely 24 h after delivery. Formation of lobulo-alveolar structures in the mammary gland also coincided with increased concentrations of estrogen in plasma during pregnancy (Delouis et al., 1980). Formation of lobulo-alveolar structure in mammary glands of heifers was completely developed when receiving estradiol-17B and progesterone (Croom et al., 1976). Estrogen injection in ovariectomized adult mice stimulated DNA synthesis in mammary ducts (Bresciani, 1968). McManus and Welsh (1981) also found that estrogen administration stimulated DNA synthesis in mouse ductal epithelium.

A very clear negative correlation between plasma progesterone and lactogenesis has been reported (Denamur,

1971 ; Kuhn, 1968). In rats, plasma progesterone concentrations reached a maximum on about the 15th day of gestation. A subsequent decline preceded initiation of lactose synthesis on days 19-21 (Kuhn, 1968). Proliferation of mammary epithelium and lobulo-alveolar morphogenesis during pregnancy clearly require progesterone (Bresciani, 1968). While progesterone did not appear to be required for ductal elongation, development of alveoli was dependent upon progesterone in the mouse (Freeman and Topper, 1978). Bresciani (1968) also reported that in ovariectomized adult virgin mice, progesterone stimulated DNA synthesis in ductal epithelium. These observations suggest that progesterone can have effects that are independent of estrogen. However, enhancement of DNA synthesis was observed when estrogen and progesterone were administered together (Haslam and Shyamala, 1979). High plasma levels of progesterone present during gestation prevented premature lactation (Kuhn, 1969). However, milk secretion was initiated experimentally by reducing plasma progesterone level. Ceriani (1970), using organ culture, reported that progesterone either alone or with estrogen produced no effect on mammary epithelium of embryonic rat tissue. However, progesterone in combination with insulin and prolactin enhanced ductal proliferation. Edery et al. (1984) also found that an increase in cell proliferation was observed when normal rat mammary epithelial organoids were cultured within a collagen-gel matrix. However, a three-to -four fold increase in cell

number was observed with prolactin plus progesterone. Thus, prolactin may mediate the progesterone effect.

Amenomori et al. (1970) reported that plasma levels of prolactin in the rat are comparatively high on the 2nd and 3rd day of pregnancy, decrease until the 20th day and then rise significantly on the 22nd day. Delouis et al. (1980) reported that prolactin was required for formation of the lobuloalveolar structures of the mammary gland. Prolactin plays a key role in mammary gland differentiation by favoring mammary growth, initiating milk synthesis and in many species maintaining established lactations (Houdebine et al., 1985).

Denamur (1971) reported that increases in plasma estrogen and prolactin were correlated with the onset of milk secretion. During pregnancy, progesterone partially inhibited stimulatory effects of estrogen on prolactin synthesis (Chen and Meites, 1970). Thus, it seems likely that the fall in progesterone and rise in estrogen in plasma are key factors responsible for the secretion of prolactin observed at the end of pregnancy in the rat (Denamur, 1971). Vonderhaar (1984) demonstrated in ovariectomized mature virgin mice, that extensive lobuloalveolar development occurred, when prolactin was administered at 2 mg/day for 1 wk along with estrogen and progesterone. This hormone combination resulted in a gland that was morphologically similar to that of a midpregnant mouse. Prolactin administration alone, however, had little or no effect on

lobuloalveolar development in mice. In contrast, Ray et al. (1955) reported that injection of saline extracts of rat placenta into ovariectomized rats stimulated lobuloalveolar development considerably. Recently, Thordarson et al. (1989) reported that the DNA content of the mouse mammary gland was not affected by hypophysectomy when estimated on day 14 and 18 of pregnancy, probably because of the presence of placental lactogen in high concentration during this period. Simpson and Schmidt (1971) reported that intraductal injection of prolactin in pregnant rabbits induced both secretory activity and cell proliferation.

Prolactin receptor numbers in mammary tissue increase with onset of lactation in most species (Nagasawa et al., 1979). Specific actions of prolactin on mammary epithelial tissue include effects on ion transport (Falconer and Rowe, 1977), amino acid transport (Pocius et al., 1980), Golgi volume (Akers et al., 1981), RNA and casein synthesis (Servely et al., 1982).

Davis and Liu (1969) demonstrated that adrenalectomy on the 14th or 15th day of pregnancy in rats inhibited subsequent secretory activity. Expansion of the mammary alveoli, diminished considerably the secretion of casein-like material, and slowed the increase in mammary RNA. However, cortisol or corticosterone administration restored normal milk secretion in adrenalectomized and ovariectomized rats, which confirmed that glucocorticoids are essential for lactogenesis in the rat. In alveolar cells, cortisol

induces differentiation of the rough endoplasmic reticulum and Golgi apparatus (Mills and Topper, 1970).

Tucker and Meites (1965) indicated that glucocorticoids given to cattle with well-developed mammary glands quickly initiated secretion of milk. Houdebine et al. (1985) reported that glucocorticoids appeared to potentiate prolactin action for induction of milk synthesis and for accumulation of milk protein mRNA, but are inactive alone. Mehta et al. (1980) reported that mouse mammary glands cultured in vitro in the absence of glucocorticoids were insensitive to prolactin stimulation. Ray et al. (1981) also found that isolated mouse mammary cells cultured for long times without glucocorticoids became strongly dependent on steroid hormones for synthesis of α-lactalbumin. Hence, cell growth in the absence of glucocorticoids is less responsive to prolactin stimulation, and high levels of cortisol increase prolactin release (Sar and Meites, 1968).

The need for metabolic hormones such as insulin in experimental stimulation of mammary gland growth was emphasized by Jacobsohn (1961). Kumaresan and Turner (1965b) indicated that when rats were treated with alloxan, which virtually destroys all insulin-producing β-cells of the pancreas, and given estrogen plus progesterone for 19 d, mammary gland mass was reduced 18% below control rats. Moreover, rats given 3 units of insulin per day plus estrogen and progesterone after alloxan treatment had 66% more mammary DNA than controls. In pregnant rats, insulin

plus growth hormone resulted in a 62% increase in mammary DNA concentration over control animals (Kumaresan and Turner, 1965b). Flint et al. (1979) demonstrated that insulin increased utilization of glucose by the mammary gland and decreased utilization of glucose by adipose tissue for lipid synthesis (Collier et al., 1984). Thus, available glucose is shunted preferentially towards mammary metabolism.

Thyroid hormones, in concert with prolactin, play an important role in regulation of development of the mouse mammary gland. Decreased levels of thyroid hormones in serum-free culture medium retarded growth of the ductal system and resulted in little or no alveolar development (Vonderhaar and Creco, 1979). However, the mammary gland is fully capable of differentiation in vitro when exposed to the proper level of thyroid hormone. When mammary explants from mice in midpregnancy were cultured in a medium containing prolactin, cortisol and insulin, a marked stimulation of the casein synthesis and activity of α-lactalbumin occurred. When thyroid hormones (T_3 or T_4) were added to the serum-free culture medium containing prolactin, cortisol and insulin, there was a marked stimulation of α-lactalbumin activity (Vonderhaar, 1975). This is in agreement with Bhattacharjee and Vonderhaar (1984) who indicated that when physiological levels of T_3 were added to a medium containing insulin, prolactin and hydrocortisone, there was an increase in casein and α-lactalbumin synthesis

compared to those cultures in the absence of T_3. The increase in α-lactalbumin synthesis ranged from 40-100%.

The concentration of growth hormone in blood remained low during gestation and lactation in rats (Schalch and Reichlin, 1966) and cows (Oxender, 1972). Moon (1961) reported that administration of 1 mg growth hormone for 19 d in ovariectomized rats was ineffective in stimulating a significant increase in DNA. However, growth hormone stimulated a significant increase in DNA concentration when given with estradiol and progesterone. Synergistic action of growth hormone and ovarian steroids in promoting an increase in mammary DNA was confirmed also by Kumaresan and Turner (1965a). Machlin (1973) found that growth hormone injected three times per week markedly enhanced milk production of dairy cows. The concentration of growth hormone in serum following administration of thyroid hormone was substantially greater in early than late lactation (Vines et al., 1977), which coincides with the highest rate of milk secretion. Thatcher and Tucker (1970) reported that injection of cortisol plus growth hormone maintained mammary cell numbers (DNA) and metabolic activity per cell (RNA/DNA) during prolonged lactation (day 32) at levels nearly comparable to rats at day 16 of lactation.

These observations indicate that growth and differentiation of the mammary gland is a complex phenomena involving a series of morphological and biochemical changes under the influence of a variety of hormones (Topper and

Freeman, 1980).

DIETARY INFLUENCES ON MAMMARY GLAND GROWTH AND FUNCTION

It is well known that changes in maternal tissues and organs which accompany reproduction are generally modified by dietary factors before and during pregnancy (Marcos and Varela, 1989). Protein deficiency or food restriction during gestation may result in reduction of postnatal weight gain, DNA, RNA, protein content, rates of synthesis and secretion of α-lactalbumin, mammary hypertrophy and hyperplasia, and a lower rate of milk production during lactation (Curtis, 1952; Baker et al., 1969; Atimo et al., 1974; Rosso et al., 1981; Geursen et al., 1987). However, gestation diet intake did not generally affect number of pigs farrowed or weaned. Reduced milk production caused by underfeeding during gestation was not reversed by excessive nutrition of the sow during lactation (Baker et al., 1969). The fact that growth of the mammary gland is directly related to milk production (Ceriani, 1974) suggests that undernutrition during gestation impaired mammary gland growth and development.

Exactly how undernutrition during gestation mediates mammary gland development is unknown. In fact, few in depth studies addressing this question have been conducted in swine. It seems that factors related to changes in secretion rates of key hormones would affect the mammary

gland. Studies in rats demonstrated that the threshold for mammary response to hormones was inversely proportional to the amount of feed intake (Trenton and Turner, 1941). Restriction of feed intake to 50% of normal reduced steroid hormone-induced mammary growth by 18% in ovariectomized rats (Srivastava and Turner, 1966a). Friedman and Reichlin (1965) indicated that somatotrophin content of the pituitary gland of rats was decreased progressively by starvation for 24-96 hrs. Rats given 75% or 50% of their normal food consumption had reduced thyroid hormone secretion rates by comparable amounts (Grossie and Turner, 1962). Another study by (Srivastava and Turner, 1966b) indicated that when 2 ug estradiol benzoate and 6 mg progesterone were injected foe 19 days into ovariectomized rats, and subjected to restriction of food to either 75% or 50% of voluntary feed intake, DNA content of the mammary gland was depressed only 4 and 18%, respectively.

In mammals, nutrients are utilized by tissues involved in maintenance, maternal tissue growth, the developing fetus, and the mammary gland during pregnancy (Bauman and Currie, 1980). Rattary et al. (1974) reported that during pregnancy, when the conceptus is growing rapidly and making maximal demands on the dam, mobilization of maternal body reserves may occur to meet demands of fetal growth. Zartarian et al. (1980) reported that protein deficiency in rats during gestation did not affect weights of fetuses or placentas, thus indicating that the fetus is "protected"

from malnutrition sufficient to impair maternal tissue function. Hence, the high priority accorded the demands of the fetus may reduce severely the nutrient supply to the mammary gland.

During gestation and lactation, profound changes occur in maternal metabolism that enable the mammary gland to supply the necessary energy and nutrients to the suckling young. Cripps and Williams (1975) observed that food intake increased 60% during pregnancy and a further 250% during lactation. Zartarian et al. (1980) reported that the total protein, RNA and DNA content of the liver increased significantly during pregnancy.

Different methods have been used for inducing malnutrition in suckling pups. Fostering large litters, separating the pup from dam, reducing the number of available teats, feeding the dam a diet low in protein, and restricting voluntary feed intake have all been assumed to result in decreased quantity and quality of milk. However, Crnic and Chase (1978) reported that weight loss and anemia were observed only in cases where dams were fed low-protein diets or were fed a reduced amount of a good quality diet. The low-protein diet lowered serum protein concentration and resulted in reduced nitrogen content and elevated total fat content of milk.

Maternal nutrition status was affected only in the cases where dams were fed a low protein, low protein quality diet (Glore and Layman, 1985). Pregnant rats showed

significant changes in body composition during the second half of pregnancy and weight gain was severely impaired in gravid rats receiving a low protein diet compared with nongravid rats (Zartarian et al., 1980). This indicated that the protein requirement for weight gain during pregnancy was greater than that for maintenence in the nongravid state.

It is clear that either protein or feed restriction during gestation and/or lactation affected adversely quality and quantity of milk. Sykes et al. (1948) reported that severe restriction of feed intake during pregnancy resulted in a 80% reduction in mammary mass, but only a 20% reduction in total body weight. Increased dietary protein quality and quantity resulted in large increases in mammary protein synthesis and pup weight, both of which are highly correlated with milk yield (Sampson et al., 1986). Grigor et al. (1987) reported that rats fed restricted amounts of protein or total diet had lower mammary gland mass and milk yield than rats fed the control diet ad libitum. However, milk yield of rats receiving the low protein diet was significantly lower than that from rats receiving restricted amounts of a control diet. This indicated that the reduction in milk production of rats receiving the low-protein diet was a result of dietary protein deprivation per se rather than of reduced food intake. This is in agreement with Sampson et al. (1986) who indicated that increasing intake of a diet deficient in protein quality and quantity

will not necessarily improve lactation performance; however, improving protein quality or quantity with or without increasing feed intake may markedly improve lactation performance.

Glore and Layman (1985) indicated that both immediate and long-term effects of a 50% restriction of food intake during lactation was more detrimental to postnatal of mammary gland growth than 50% food restriction during gestation. Chow and Lee (1964) reported that food restriction during gestation and lactation or during gestation alone, by as little as 25% of that consumed by the unrestricted group, resulted in growth stunting of the progeny, anemia, and decreased resistance to hyperthermia. Female rats receiving a diet containing 15% protein through pregnancy to 14 d of lactation had better developed mammary glands with greater amounts of DNA and RNA than rats fed a 10% protein diet (Pyska and Styczynski, 1979a). Furthermore, prepubertal food restriction reduced DNA content in the mammary gland of rats subsequently fed a 10% protein diet by 23% compared to females subsequently fed a 15% protein diet. The amount of RNA was 19% lower (Pyska and Styczynski, 1979b). This is in agreement with Rosso et al. (1981) who reported that feed restriction from day 5 of gestation markedly reduced mammary gland weight compared to control, ad libitum fed animals. Affected glands also had a significant reduction in DNA, RNA and protein content. Thus, restriction of protein intake during pregnancy affects

mammary gland development and protein synthesis, presumably by altering both cellularity and rate of synthesis per cell.

During the late gestation mammary glands of most mammals, such as rats and humans, undergoes a marked hyperplasia modulated by the combined influence of various placental hormones (Winick and Noble, 1964). Zartarian et al. (1980), demonstrated that pregnant rats underwent extensive loss of muscle mass and protein during late gestation. Thus, restriction of feed intake during late gestation may impair rat mammary gland hypertrophy and hyperplasia (Rosso et al., 1981).

Increased protein intake did not affect the composition of milk, but did increase milk yield (Mueller and Cox, 1946). Concentration of total lipid, total protein and lactose in milk was not affected by feed restriction or by feeding of low protein diets. However, the concentration of α-lactalbumin in milk of rats fed a low protein diet was significantly lower than in milk of rats fed a control diet, irrespective of feed intake (Grigor et al., 1987). Grimble (1981) reported that low quality proteins in the maternal diet resulted in a 20% decrease in total milk protein, whereas milk fat, lactose and free amino acid nitrogen were unaffected. In a study using acini isolated from mammary tissue by collagenase digestion, Geursen et al. (1987) found that feeding rats a low protein diet (10% casein), or restricting intake of the control diet to 40% of voluntary intake, resulted in significantly lower rates of synthesis

and secretion of α-lactalbumin and casein, but not the rate of synthesis of fatty acid synthase compared to rats consuming a high protein (20% casein) or control diet ad libitum. Furthermore, starving rats for 48 h resulted in significantly lower rates of synthesis and secretion of both α-lactalbumin and fatty acid synthase. However, subsequent refeeding for 48 h restored the rates of synthesis and secretion of α-lactalbumin, casein and fatty acid synthase to near control values within 24 h (Geursen et al., 1987).

The mammary gland is by far one of the major sites of nitrogen utilization in lactating animals (Mepham, 1982). Davis and Collier (1985) reported that lactating mammary glands are dependent upon its blood supply to provide substrates at appropriate rates for milk synthesis. The rate of mammary supply of substrates is determined by substrate concentration in blood and rate of mammary blood flow (Mepham, 1982). During parturition, mammary blood flow and cardiac output increased substantially, and mammary blood flow also increased in proportion of cardiac output (Linzell, 1974). Thus, restriction of substrate supply to the mammary gland may influence milk synthesis. When examined cardiac output, organ blood flow and organ weights in rats restricted to 50% of the intake of control dams. Sakanashi et al. (1987) found that, during lactation, control dams gained weight whereas acute restricted dams lost weight. Milk yield among acute restricted dams was 58% lower than the control dams. Cardiac output in the

restricted dams was 57% of that in the control group. Mammary gland blood flow rate and weight were reduced in acute restricted dams. Experiments with isolated perfused mammary glands (Davis and Collier, 1985) indicated that essential amino acid uptake into the mammary gland could be divided into 2 groups, those for which uptake equals output in milk protein (methionine, tyrosine, phenylalanine, histidine and tryptophan), and those which were apparently absorbed in excess of milk protein output (threonine, valine, isoleucine, leucine and arginine). Utilization of amino acids is comprised of two stages, uptake of blood amino acids by secretory cells and intracellular metabolism of these amino acids. Once amino acids enter the cell, most will undergo RNA directed polymerization to form milk proteins. A portion will enter into metabolic reactions yielding polyamines and nonessential amino acids (Mepham, 1982). Studies with rats showed that short-term starvation decreased amino acid supply and transport in mammary gland as well as free amino acid concentrations in milk (Vina et al., 1987). Jansen et al. (1986) reported that as dietary protein concentration increased from 12% to 22%, milk yield increased threefold, free lysine concentration six- to eightfold and methionine concentration increased two to threefold. Infusion of essential amino acids in isolated guinea pig mammary gland led to an apparent 27% increase in protein synthesis (Peters et al., 1979). Thus, essential amino acids appear to play a key role in mammary gland

development. Omission of a single essential amino acid is known to have adverse effects on pregnancy (Niiyama et al., 1973).

Arginine is considered a nonessential amino acid in numerous mature mammals including humans (Rose et al., 1954), rats (Burroughs et al., 1940) and pigs (Baker et al., 1966). Milner and Visek (1978a) reported that when growing rats were fed an arginine-deficient or arginine free diet, urinary citrate and orotic acid excretions were elevated. In contrast, Carey et al., (1987) reported that when arginine was removed from the human diet, urinary orotic acid did not increase. However, this study was only conducted for a 5 d period. Easter and Baker (1976) concluded that consumption of an arginine-free diet during pregnancy by gravid gilts did not affect fetal development. This indicated that in vivo synthesis of arginine was probably sufficient to meet both maternal and fetal needs. Milner and Visek (1978b) reported that urinary urea, citric acid and orotic acid concentrations, which are indicators of arginine deficiency, increased during gestation when a 12% casein basal diet was fed. Addition of 1% dietary arginine reduced citric acid excretion. Furthermore, arginine supplementation reduced orotic acid excretion during late gestation and early lactation which indicated that the rate of endogenous arginine synthesis failed to meet the arginine requirement during gestation and lactation. However, they did not study either mammary gland development or lactation

performance. Pau and Milner (1981,82) observed that feeding arginine-deficient diets or feeding control diets at 50% of ad libitum intake decreased mammary gland weight, total DNA and RNA content of gravid rats, but no change in RNA:DNA ratio was observed. These studies suggest that feeding an arginine-free diet to gravid rats markedly impairs mammary gland development by decreasing mammary gland mass and nucleic acid content.

Uptake of arginine is in excess of output in milk protein for the mammary gland of the goat (Mepham and Linzell, 1966) and sow (Linzell and Mepham, 1969). Peters et al. (1979) indicated that when guinea-pig mammary gland was infused with the essential amino acids threonine, valine, isoleucine, leucine and arginine, uptake of all these amino acids was stimulated significantly, however, leucine and arginine uptakes showed the largest increases.

Results of an earlier study (Folley and Greenbaum, 1946) suggested that the rat lactating mammary gland exhibited appreciable arginase activity and that, in fact, it was probably the second richest source of arginase in the body. In rats, mammary gland arginase activities increased slowly during pregnancy and early lactation and increased dramatically between the 5th and 10th days of lactation. Arginase reaches a maximum at the 20th day of lactation and after weaning fells precipitously to early pregnancy concentration (Folley and Greenbaum, 1947). Greengard et al. (1970) reported that arginase was found in fetal liver

and later increased to a high concentration in adult liver. Furthermore, arginase was also found in small but significant concentrations in small intestine, kidney and pancreas. Arginase activity in the mammary gland increased from zero in the virgin rat mammary gland to substantial activity during pregnancy and lactation (Greengard et al., 1970). Even though arginase was found in mammary tissue, the urea cycle enzyme, arginosuccinate synthetase, could not be detected, thus indicating an incomplete urea cycle in mammary tissue (Yip and Knox, 1972).

Research conducted by Verbeke et al. (1968) indicated that when lactating mammary glands of sheep were perfused for several hours in the presence of DL-(2-^{14}C)ornithine or DL-(5-^{14}C)arginine, about 13% and 9% of milk casein proline was derived, respectively, from plasma free ornithine and free arginine. They further concluded that since use of arginine as a substrate for proline synthesis proceeded via ornithine, conversion of arginine to ornithine was probably catalyzed by arginase; hence, arginase may be involved in the process of milk synthesis. Yip and Knox (1972) demonstrated that activities of arginase and ornithine aminotransferase, rate limiting enzymes for proline synthesis, increased in mammary gland during gestation, further increased during lactation, and subsequently declined during postweaning mammary involution.

Excess uptake of arginine by the mammary gland seems to be important for one or more products of metabolism (Mepham,

1982). It is likely that the significance of arginine lies in its role as a precursor of proline in the mammary gland of sheep and goats (Roets et al., 1974), cows and rabbits (Clark et al., 1975) and rats (Mezel and Knox, 1977). Arginine may also serve as a precursor of proline in sows, since the uptake of proline into sow lactating mammary glands is apparently negligible (Linzell and Mepham, 1969).

ROLE OF POLYAMINES IN MAMMARY GLAND

In mammalian cells, the biosynthesis of polyamines, putrescine, spermidine and spermine, involves two decarboxylases and two aminopropyltransferases (Tabor and Tabor, 1976). The first step is the decarboxylation of L-ornithine to putrescine catalyzed by the pyridoxal phosphate dependent enzyme L-ornithine decarboxylase (ODC) (Russell and Synder, 1968). The second carboxylase S-adenosyl-L-methionine decarboxylase (SAMDC) which generates an active aminopropyl group needed to convert putrescine into spermidine and spermidine to spermine (Pegg and Williams-Ashman, 1970).

Arginine is a precursor of spermidine and spermine, which may be more important than its role as a precursor of proline. Through the action of arginase, significant quantities of ornithine are formed, yet ornithine is not present in milk (Clark et al., 1975). This indicates that ornithine is either oxidized completely or metabolized to

proline, citrulline or putrescine in the mammary gland. Formation of polyamines from arginine has been demonstrated in polyamine-requiring mutants of Chinese hamster ovary cells (Pohjanpelto et al., 1981), in which defective arginase inhibits de novo synthesis of putrescine. Bedford et al. (1987) reported that increased dietary lysine increased significantly renal arginase activity in chicks, and reduced renal ODC activity. Increased dietary lysine also caused an increase in putrescine and ornithine concentrations. This indicates that accumulation of polyamine precursors results in increased concentrations of putrescine with subsequent feedback inhibition of ODC activity. Biosynthesis of polyamines was first described by Tabor et al. (1958), who demonstrated that ornithine served as a precursor for formation of spermidine. Methionine was also a precursor of spermidine and spermine (Tabor et al., 1958; Pegg and William-Ashman, 1970). Concentrations of spermidine and spermine in cow and human milk varies with methionine intake (Sanguansermsri et al., 1974). Fausto (1971) indicated that a mixture of histidine, lysine and arginine injected into rats caused increased liver ODC activity to levels equal to, or higher than, those obtained with casein administration. Furthermore, when these amino acids were injected individually, lysine proved to be ineffective in eliciting ODC activity changes, but either histidine or arginine produced very marked increases in ODC activity. Hence, it appears that alteration of intake or

metabolism of arginine and/or methionine may alter polyamine biosynthesis in the mammary gland.

Although polyamines exist in cells in millimolar concentrations and their biosynthesis appears to be related intimately to cell division and growth, they are among the few remaining metabolites about whose function we have little solid information. It has been suggested that polyamine synthesis in various tissues is primarily dependent upon the way in which nutrients are made available to the tissues (McAnulty and Williams, 1977). When fasted rats were fed a 50% casein diet, ODC activity and polysome-associated ODC-mRNA activity increased 50-to-100 fold. In contrast, zein (low in tryptophan and lysine), gelatin (low in tryptophan and methionine) and hemoglobin (low in isoleucine) did not induce ODC responses unless the diet was supplemented with appropriate amino acids (Kameji et al., 1987). This indicated that an adequate supply and balance of amino acids are required to induce hepatic ODC activity (Kameji et al., 1987). McAnulty and Williams (1977) reported that when rats were undernourished, ODC activity decreased in liver. Refeeding for 4 h significantly increased ODC activity. Similar results were observed after refeeding rats with high protein diets (60% casein) for 2 h after starvation for 22 h (Pariza et al., 1976). These reports indicate that phenotypic expression of ODC activity in liver and kidney tissue of adult animals is responsive to extremes of starvation and refeeding. Induction of ODC

activity was correlated with increased liver cell proliferation in response to refeeding (Russell and Synder, 1968). However, Hawrylewicz et al. (1989) demonstrated that unstressed, normal, young growing rats fed a high protein diet (33% casein) to 7 wk of age had significantly higher ODC activities in liver than rats fed a normal protein diet (19% casein). When rats were switched from the normal to the high protein diet, ODC activity increased significantly. This suggests that the increase in liver ODC activity is responsive to changes in dietary protein. This observation is in agreement with Farwell et al. (1977) who demonstrated that feeding a protein-free diet followed by a high casein diet caused a significant increase in ODC activities in the liver and kidney. Spermidine concentration was increased approximately 50% in the liver. Induction of ODC activity in mammary glands via starvation and refeeding has been reported also. Brosnan et al. (1983) reported that starvation markedly increased putrescine content in the mammary gland of lactating rats and a marked decrease in ODC activity. However, refeeding for 5 h caused ODC activity and spermidine and spermine concentrations returned to normal fed levels. Phenotypic change in ODC activity in the liver via high protein feeding has also been reported recently by Hawrylewicz et al. (1989) in rat mammary glands. They found that, at 7 wk of age, the ODC values for mammary epithelial cells from rats fed a high protein diet was significantly greater than that of rats fed a normal protein

diet. Furthermore, rats switched from the high- to normal-protein diet had lower mammary epithelial ODC activity than did the high protein group. Conversely, changing from the normal-to high-protein diet tended to result in slightly higher ODC activity than the control group. These results indicate that increases in dietary protein concentration may stimulate RNA and protein biosynthesis in mammary epithelial cells of the developing mammary gland, since ODC activity has been implicated as an early response of cellular proliferation.

Pregnancy in mammals causes profound morphological and functional modifications in maternal organs; increases polyamine urinary excretion and concentration in maternal plasma and in organs of the reproductive system; and elevated ODC and SAMDC activities in these organs (Kobayashi, 1964; Porta and Pietra, 1981). Piacentini et al. (1986) demonstrated that rat liver ODC activity increased 4.5 to 5 fold on day 15 to day 17 of pregnancy. Putrescine and spermidine concentrations increased 3 to 4 fold, but no variation in spermine content was observed. Furthermore, no significant variation in ODC activity or spermidine and spermine concentrations were observed in kidneys, whereas putrescine concentration showed significant variation throughout pregnancy. Lundgren and Oka (1978) reported that the concentration of spermidine in rat blood increased significantly by day 6 of pregnancy, and the largest increase occurred shortly before parturition. After

birth, spermidine decreased, increased again at the 3rd day of lactation, and declined subsequently to concentrations found in the virgin rat. Moreover, the concentration of spermine remained constant throughout the whole period. Furthermore, ODC activity increased asynchronously in liver and kidney of pregnant and lactating rats during times when circulating spermidine concentrations were elevated (Lundgren and Oka, 1978). Yang et al. (1984) reported that during the first half of lactation, mucosal ODC activity increased, with the maximal increase occurring on day 5 which corresponds to the time of maximal morphological changes in the intestine during lactation. These results suggest that ODC activity plays an essential role in kidney, liver and intestinal hypertrophic and hyperplastic growth during adaptation to pregnancy and lactation.

Variation in ODC activity and polyamine concentration have been observed also in rat mammary glands during gestation and lactation. In studies using rats, Russell and McVicker (1972) reported that activities of ODC and SAMDC, the two rate-limiting enzymes for the biosynthesis of polyamines, increased in middle and late pregnancy, and further increased during lactation. Moreover, key lactogenic hormones, insulin, prolactin and glucocorticoid, promote increased levels of these two enzymes and arginase (Oka and Perry, 1974a). This was confirmed with Rillema et al. (1977) who demonstrated that spermidine concentration, arginase and ODC activity were elevated when mammary gland

explants were incubated with hydrocortisone, insulin and prolactin. Sakai et al. (1978) reported that insulin elicited sequential stimulation of the activity of ODC, SAMDC and spermidine synthase, and increased the concentration of putrescine and spermidine prior to augmentation of DNA synthesis in mammary gland explants.

Moderate concentrations of spermidine and spermine have been found in cow and human milk (Sanguansermsri et al., 1974). Russell and McVicker (1972) indicated that spermidine and spermine act to increase the effective activity of RNA polymerase, thus suggesting that synthesis of rRNA during growth should precede an increase in polyamine synthesis. In addition, the ratio of spermidine:spermine concentrations appears to be an excellent indicator of mammary gland synthetic activity. An increase in this ratio from one to ten upon initiation of lactation in rats signals increased biosynthetic capacity for RNA and protein.

Concentrations of spermidine and putrescine in the mouse (Oka et al., 1978) and rat (Russell and McVicker, 1972) mammary gland remained low in the virgin state when mammmary cells are developmentally dormant. Increase in spermidine concentration was increased two- to three-fold during pregnancy and six- to eight-fold during lactation, while the concentration of putrescine increased five-fold and two-fold, respectively, during the same periods. However,no significant changes in spermine concentration was

found during these period (Oka et al., 1981). These results indicate that polyamines may be involved in the proliferation of mammary epithelial cells. Sakai et al. (1978) demonstrated that addition of methyl-glyoxal-bis-guanylhydrazone (MGBG) (a potent inhibitor of spermidine biosynthesis) and insulin to the medium at the onset of culture completely prevented increases in both the concentration of spermidine and DNA synthesis. Addition of spermidine restored intracellular concentration of spermidine to control levels within 4 h, and DNA synthesis in about 10 h. The same study by Sakai et al. (1978) showed that inhibition of DNA synthesis by MGBG was also reversed by addition of exogenous putrescine and spermine.

The role of polyamines in milk protein synthesis has been investigated. When mammary tissue from mid-pregnant mice was cultured with insulin, cortisol and prolactin, mammary cells synthesized casein and α-lactalbumin (Topper and Oka, 1974). Oka and Perry (1974c) reported that prior to the accelerated synthesis of milk proteins, the concentration of spermidine increased significantly, amounting to about a 3-fold increase at 48 h, the time at which milk protein synthesis was maximal. However, when MGBG was added to the culture, hormonal stimulation of both the concentration of spermidine and accumulation of casein and α-lactalbumin was blocked (Oka and Perry, 1974c). Inhibitory effect of the drug on milk protein synthesis was reversed completely by addition of spermidine, but not by

spermine or putrescine. Similar results were obtained by Rillema et al. (1977). These results suggest that spermidine may be necessary for milk protein synthesis. Another study conducted by Oka and Perry (1974a) demonstrated that when mammary explants were cultured in medium in which the concentration of arginine was reduced from 4 mM to 0.2 mM and synthesis of spermidine and α-lactalbumin was inhibited. However, addition of ornithine, putrescine or spermidine restored α-lactalbumin synthesis; whereas the addition of spermine, proline or lysine did not affect α-lactalbumin synthesis. These results suggest strongly that putrescine or spermidine may be involved in milk protein synthesis.

Addition of spermidine, in place of cortisol, to a media containing insulin and prolactin, markedly enhanced milk protein synthesis (McCarty and McCarty, 1975). The increase in α-lactalbumin was similar to that produced by the combination of insulin, cortisol and prolactin. However, the increase in casein synthesis was about half of that observed in the three-hormone system. Spermidine alone, or in combination with any one of the three hormones or with both insulin and cortisol, was ineffective (Oka, 1974). This effect of spermidine appeared to be specific for glucocorticoids. Additional studies conducted by Oka and Perry (1974b) demonstrated that, in mammary cells cultured only with insulin, activity of glucose-6-phosphate dehydrogenase, an enzyme that plays an important role in

lactogenesis, increased 50-100% during the early period of culture, and then declined by day 3 and 4. Addition of glucocorticoids or spermidine prevented the decline, thus suggesting that spermidine mediates several important functions of glucocorticoids during differentiation of mammary epithelial cells.

The diet fed during gestation is an important factor in producing satisfactory lactation results during lactation (Fairbanks et al., 1945) However, knowledge on how lactational performance may be improved or altered through manipulation of gestational diets is still unclear. Omission of arginine during gestation in rats markedly impairs mammary gland development by decreasing mammary gland mass and nucleic acid content. The concentration of polyamines in rat mammary glands remained low in the virgin state, and rose during pregnancy as proliferation of mammary epithelial cells occurred (Russell and McVicker, 1972). Tabor and Tabor (1984) reported that arginine serve as a precursor of polyamines. Alteration of arginine may induce ODC activity and polyamine synthesis which are involved in proliferation of mammary epithelial cells. Thus, it is important to study the requirement and effect of dietary arginine during gestation, and the role of arginine on mammary gland development.

Objectives of the current studies were: 1) to determine the arginine requirement of nongravid and gravid rats during gestation; 2) to examine the effect of arginine on

reproductive performance and mammary gland development; 3) to examine the effect of dietary arginine during early and late gestation on rat mammary gland development; and 4) to examine possible relationships of dietary arginine and polyamine biosynthesis with mammary gland development.

CHAPTER III

MATERIAL AND METHODS

ANIMALS, GENERAL ASSAY AND MANAGEMENT

Sexually mature virgin Fisher F344/NTacfBR rats (Taconic Laboratory Animals and Services, Germantown, NY) at least 8 weeks of age were used in all experiments. In experiment 1, rats began on treatment at an average body weight of 160 g; in experiments 2-4, rats averaged 191 g body weight upon initiation of treatments. Animals were kept at 20-22°C under 12 h light and 12 h dark photoperiod and fed ad libitum with a commercial diet (Ralston Purina Co., St. Louis, MO). Rats were transferred to separate cages for mating. The following day, the presence of a vaginal plug was considered as evidence of mating. The vagina was rinsed with saline and the presence of vaginal sperm was considered evidence of pregnancy. A modification of the basal rat diet described by Robbins et al. (1980) was used and is presented in Table 1. The basal diet was formulated to meet the NRC (1978) guidelines for all nutrients except arginine. Percentage of dietary arginine ranged from 0.0% to 0.75%. All diets were formulated to contain 1.98% N and were made isonitrogenous to the control diet by appropriate variations in glycine. Additions to the basal diets were made at the expense of sucrose.

Table 1

Composition of purified crystalline amino acid basal diet

Basal diet	%	Amino acid mix	%
Sucrose	to 100.00	L-arginine.HCl	0.0–0.75
Dextrin	48.00	L-histidine.HCl.H_2O	0.45
Amino acid mixture	12.85–13.60	L-lysine	1.40
Corn oil	5.00	L-tyrosine	0.35
Mineral mixture[1]	5.11	L-threonine	0.82
Vitamin mixture[2]	0.20	L-tryptophan	0.18
Sodium bicarbonate	1.00	L-phenylalanine	1.16
		DL-methionine	0.82
		L-cystine	0.30
		L-leucine	1.00
		L-isoleucine	0.82
		L-valine	0.82
		Glycine[3]	2.33
		L-proline	0.35
		L-asparagine.HCl	0.60
		L-alanine	0.35
		L-aspartic acid	0.35
		L-serine	0.35
		L-glutamic acid	0.40
		Total	12.85–13.60

[1] Mineral mixture, as percent of the diet: $CaCO_3$, 0.3; $Ca_3(PO_4)$, 2.8; K_2HPO_4, 0.88; $MgCO_3$, 0.09; $MnSO_4.H_2O$, 0.065; ferric citrate, 0.05; $ZnCO_3$, 0.01; $CuSO_4.5H_2O$, 0.002; $Na_2MoO_4.2H_2O$, 0.009; KI, 0.004; $CoSO_4.7H_2O$, 0.001; Na_2SeO_3, 0.00002; total 5.11%.

[2] Vitamin mixture, mg per kg of the diet: thiamin.HCl, 100 mg; niacin, 100 mg; riboflavin, 16 mg; Ca-pantothenate, 20 mg; vitamin B_{12}, .02 mg; pyridoxine.HCl, 6 mg; biotin, .6 mg; folic acid, 4 mg; inositol, 100 mg; PABA, 2 mg; menadione, 5 mg; ascorbic acid, 250 mg; DL-α-tocopheryl acetate, 20 mg; cholecalciferol, 600 ICU; retinyl acetate, 10,000 IU; total .20%.

[3] The arginine-free diet contained 0.0 % L-Arg.HCl and 4.26 % Gly.

All rats were weighed and euthanized within 8 h after parturition. To measure the activity of ODC and SAMDC in rat mammary glands, fresh tissue was removed and weighed then homogenized in an equal volume (assuming 1 g tissue = 1 ml) of ice cold homogenizing medium (described later) with a Polytron homogenizer. One ml of homogenate was frozen for protein and arginase determinations, and the remaining homogenate was centrifuged for 10 min at 10,000 x g. L-ornithine decarboxylase and S-adenosyl-L-methionine decarboxylase were assayed as described by Lowkvist et al., (1985). The method is based on trapping of $^{14}CO_2$ released from DL-1-^{14}C-ornithine in the presence of a saturating level of L-ornithine. Homogenate (100 µl) containing 0.5 mM Na_2 EDTA, 5.0 mM dithiothreitol, and 0.05 mM pyridoxal 5'-phosphate in 10 mM Tris/HCl buffer (pH 7.2) was added to 900 µl of incubation medium (pH 7.2) containing 9.0 µmol of Tris/HCl buffer, 5 µmol of dithiothreitol, 0.2 µmol of pyridoxal 5'-phosphate, 4.31 µmol of DL-1-^{14}C-ornithine, and 1.0 µmol of L-ornithine. The final specific activity of DL-1-^{14}C-ornithine was 9.21 mega becquerel/mmol. The reaction was carried out in 15-ml centrifuge tubes equipped with rubber stoppers. The $^{14}CO_2$ released during a 60 min incubation at 37.5 °C was trapped in 0.1 ml Hyamine hydroxide contained in a plastic well attached to the rubber stopper with a wire. The enzymatic decarboxylation was stopped by injection of 1 ml of 40% trichloroacetic acid and the incubation was continued for 30 min to ensure that all $^{14}CO_2$

was released from the incubation mixture and was trapped in the Hyamine hydroxide. The well and its contents were then be removed and placed in a scintillation counting vial containing 5 ml of CytoScint scintillation solution (ICN Biomedicals, INC., Irvine, CA). Samples were counted in a Beta scintillation counter (Searle, Elk Grove Village, IL.) for 1 min.

The SAMDC assay was based on capture of $^{14}CO_2$ released from S-adenosyl-L-(carboxyl-^{14}C)-methionine. A 50 µl aliquot of homogenate containing 0.1 mM Na_2EDTA and 5 mM dithiothreitol in 0.05 M sodium-potassium-phosphate buffer (pH 7.2) was added to 470 µl of incubation medium (pH 7.2) containing 4.0 µmol Tris/HCl, 2.5 µmol of dithiothreitol, 25 µmol of putrescine, and 6.14 µmol of S-adenosyl-L-(carboxyl-^{14}C)-methionine (specific activity, 2.11 giga becquerel/mmol). Carbon dioxide released from the incubation mixture was trapped in Hyamine hydroxide. The well and its contents were counted in a Beta scintillation counter for 1 min.

The homogenate from ODC assay was used to determine the arginase activity by the procedure described by Smith and Lewis (1963) with necessary modification. Homogenate (0.1 ml) was added to 0.4 ml of homogenizing buffer and was incubated for 60 min at 37 C. After a 60 min pre-incubation, 2 ml of the 0.425 M L-arginine substrate solution (pH 9.5) was added to the reaction tube. At the end of the incubation period (exactly 2 min), the reaction

was stopped with 1 ml 1.8 M HPO_3 solution. After standing for 15 min to allow uniform protein precipitation, the tubes were centrifuged. Two ml of the supernatant fluid and 0.5 ml color reagent (3% alpha-nitrosopropiophenone in 95% ETOH) were added to 10 ml of sulfuric acid-phosphoric acid-water (0.09:0.27:0.64, v/v/v) and heated in boiling water bath for 1 h. Tubes were removed and cooled in dark. Absorbance was read on a spectrophotometer (Perkin Elmer) at 540 nm.

Protein concentration was determined using the Lowry (1951) method. Tissue homogenate (0.1 ml) from the enzyme assay was diluted to 1 ml with water. To each tube of homogenate, 1 ml of Lowry Reagent solution was added. The tubes were then vortexed and allowed to stand at room temperature for 20 min. After standing, 0.5 ml of Folin and Ciocalteu's Phenol Reagent Working solution was added with immediate and vigorous mixing. Following a 30 min reaction time, absorbance was read on a spectrophotometer at 500 nm.

The nucleic acid fraction of the mammary gland was extracted according to the method of Ogur and Rosen (1950). A weighed amount of mammary tissue was homogenized in 10 volumes of absolute ethanol (assuming 1 g = 1 ml) and then certrifuged at 12,000 x g for 20 min at 2-4°C. The solid residue was again extracted with absolute ethanol followed by two extractions with ether to remove lipids. The fat-free residue was subsequently dried completely in a desiccator for further analysis. After determination of the dry weight, the dried, fat-free tissue was extracted twice

with 2% perchloric acid at 4 C for 20 min. The remaining solid residue was then extracted twice with 10% perchloric acid at 70 C for 20 minutes to obtain the nucleic acid fraction. Perchloric acid (PCA) extracts were combined and used for determination of RNA and DNA.

Determination of RNA was based on the technique described by Ceriotti (1955). Orcinol (200 mg) was dissolved in concentrated HCl. Ten ml of 0.0004 M $CuCl_2.H_2O$ in concentrated HCl solution was added and diluted to 100 ml with concentrated HCl. Color reagent (2 ml) was added to 2 ml of the 10% PCA extract. The mixture was then incubated in boiling water for 40 min and then cooled under running water. From each reaction tube, nonspecific color was extracted with 5 ml of isoamyl alcohol and removed by aspiration of the alcohol mixture. The absorbance of the remaining mixture was determined at 675 nm. A standard curve was prepared from Bakers Yeast RNA (Sigma, St. Louis, MO).

Concentration of DNA was determined according to the method of Hubbard et al. (1970). Calf thymus DNA (Sigma, St. Louis, MO) was dissolved in 0.001 N NaOH for standard curve determination. To 2 ml of 10% PCA extract, 2 ml of color reagent (0.08% indole and 4 N HCl, v/v) was added. The tubes were mixed and heated in boiling water for 15 min. Following incubation, the tubes were cooled to room temperature in an ice bath. Nonspecific color was extracted 3 times with 4 ml of chloroform with the chloroform layer

discarded after centrifugation each time. The absorbance of aliquots of the aqueous phase were read at 490 nm.

Experiment 1:

The objective of this experiment was to determine the arginine requirement of nongravid rats. Twenty virgin rats weighing 160 g were used. Four rats were assigned randomly to one of 5 dietary treatments for 20 d. Treatments were as follows: 1) arginine free: 2) 0.15% Arg; 3) 0.35% arginine; 4) 0.55% arginine; and 5) 0.75% arginine. Basal diets are shown in Table 1.

To facilitate urine and feces collection, 5 metabolism cages were utilized. One randomly selected rat from each treatment was used for N-collection at d 1-5. A second, third and fourth rat from each treatment was used for N collection at d 6-10, d 11-15 and d 16-20, respectively. Water was available ad libitum. The sum of the N collected from 4 rats within each treatment across time were then used as the estimate of total N retention. During the collection period, feces were collected daily. One ml of 6 N HCl were added to feces then dried in an oven at 105 C for 48 h. Feces were then air-dried, mixed and ground with pestle and mortar, and filter through 1 mm screen. The sample was then saved for analysis. Urine was collected daily for each 5 d collection period. Two ml of 6 N HCl were added daily to the collecting tube. Urine was filtered through glass wool into plastic bottles daily and diluted to a constant volume for each 5 d. A 50 ml aliquot was retained and stored in

the refrigerator until analyzed. During the collection period, feed intake was measured daily. Body weight was determined weekly. The nitrogen content values of feed, feces and urine samples were determined by using the standard Kjeldahl method (AOAC, 1975). Body weight gain and feed intake were measured.

The broken line regression method (Robbins et al., 1979) was used to estimate the arginine requirement from growth data of experiments 1 and 2. The broken line consists of two parts: a straight line with an increasing slope, which represents the substantial response to increases in a limiting nutrient; and either a horizontal line or a straight line of significantly lower slope, which represents the minimal response to increases in a nutrient above the requirement. The abscissa of the point at which the two lines intersect is the estimated requirement. The broken line was fitted to body weight gain and nitrogen retention method (SAS, 1982), and yielded least squares, best fit estimates.

Dependent variables for this experiment were body weight gain and N retention. Data were analyzed statistically by the general linear models (GLM) procedure of the SAS (1982) Institute Inc. Orthogonal contrasts were used to test for linear and quadratic effects. Orthogonal coefficients for unequally spaced treatments were calculated as described by Carmer and Seif (1963).

Experiment 2:

The objective of this experiment was to examine the effect of arginine on reproductive performance and mammary gland development. Twenty female virgin rats weighing 160-180 g were used. Four rats were assigned randomly to one of the following 5 dietary treatments on day 1 of pregnancy: 1) arginine-free; 2) 0.15% arginine; 3) 0.35% arginine; 4) 0.55% arginine; and 5) 0.75% arginine. Water and feed were available ad libitum throughout pregnancy. Nitrogen collection was performed as described in experiment 1.

All maternal and pup weight were determined and then each euthanized with ether within 8 h after parturition. The mammary gland was dissected with a clean scalpel and then rubbed thoroughly with a Culturette cotton swab (Becton Dickinson. Cockeyville, MD). Cotton swabs were streched across brain heart infusion agar (Gibco Laboratories, Madison, WI) supplemented with 5% defibrinated sheep blood, 1% yeast extract, and 1% horse serum. Plates were incubated at 37 C, and bacterial growth on primary culture medium was identified tentatively according to colony morphologic features, hemolytic characteristics, Gram staining, and catalase test (National Mastitis Council, Inc., 1981). Mammary glands were dissected immediately from adherent skin, weighed and used for enzyme and nucleic acid determination.

Dependent variables for this experiment were net maternal weight, pup weight, feed intake, mammary gland weight, nucleic acid content and enzyme activities. Data

were analyzed statistically by analysis of variance using the GLM procedure of SAS (1982). Orthogonal contrasts were used to test for linear and quadratic effects. Orthogonal coefficients for unequally spaced treatments were calculated as described by Carmer and Seif (1963).

Experiment 3:

The objective of this experiment was to examine the effect of arginine deficient diet on reproductive performance and mammary gland development. Virgin rats weighing 180-200 g were used in this experiment. Rats were fed either an 0.15% (Low Arg) or an 0.60% (control) arginine purified L-amino acid diet on day 3 of pregnancy through parturition. The basal diet is presented in Table 1.

Water was available ad libitum. Body weight and feed intake were measured weekly. All rats were euthanized with ether within 8 h after parturition. Net maternal weight and total pup weight were measured immediately after parturition. Bacterial growth in an excised mammary tissue was determined as described in Experiment 2. Mammary gland tissue enzyme activities and nucleic acid contents were determined as described in the general assay section. Data were analyzed statistically by analysis of variance using the GLM procedure of SAS (1982).

Experiment 4:

The objective of this study was to examine the effect of dietary arginine during early and late gestation on rat mammary gland development. All rats were fed the control

diet from d 1 to d 5 of pregnancy. On d 6 of pregnancy, rats were assigned randomly to each of the following 5 dietary treatments: 1) fed arginine-free diet ad libitum; 2) fed an amount of the 0.60% arginine control diet equal to the amount consumed the previous day by rats fed the arginine-free diet; 3) fed 0.60% arginine ad libitum (control); 4) fed arginine-free diet from d 6 to d 14 then control diet through parturition; and 5) fed 8 g of the control diet per day from d 6 to d 14 then ad libitum through parturition.

Water was available ad libitum. Feed intake and body weight were measured weekly. Maternal weight and pups weight were measured immediately after parturition. All rats were euthanized with ether within 8 h after parturition. Bacterial growth in an excised mammary tissue was determined as described in Experiment 2. The mammary gland was dissected and used for enzyme and nucleic acid determination. Data were analyzed using the general linear models (GLM) procedure of the SAS (1982) Institute Inc. Significance of treatment differences was tested using orthogonal comparisons. The variances of all data except ODC activity in experiment 4 were homogeneous; hence pooled standard errors were calculated and are reported. The variances of ODC activity in experiment 4 were heterogeneous; thus analysis of variance was conducted on the log transformed data and the standard deviation of each mean were calculated and are reported.

CHAPTER IV

RESULTS

Experiment 1:

Sexually mature, virgin rats fed an arginine-free diet maintained positive nitrogen balance, but ate less feed and lost body weight during the 20 d experimental period (Table 2). As little as 0.15% dietary arginine prevented weight loss and increased N-retention nearly three fold. Based upon body weight gain and total N-retention, the dietary arginine requirement was estimated to be about 0.21%.

Experiment 2:

Body weight, feed intake and nitrogen retention of primigravid rats increased linearly with the addition of 0.15%, 0.35%, and 0.55% dietary arginine, then plateaued (Table 3). Arginine requirement for body weight gain was estimated to be 0.48±0.11%; and for nitrogen retention, 0.61±0.21%. Neither number of pups born, nor birth weight was affected by dietary arginine concentration. Two out of 20 rats tested positive for bacterial growth (Table 4). Mammary gland nucleic acid concentrations were not affected by presence of bacterial growth.

Both the absolute weight of the mammary gland, and gland weight expressed as a percent of total body weight increased linearly and significantly ($P<0.03$) with increases in dietary arginine concentration (Table 5). Total mammary

Table 2

Growth and nitrogen retention of non-gravid rats fed varied levels of dietary arginine for 20 days (Experiment 1)

	Dietary Arginine (%)					
Variables	0.0	0.15	0.35	0.55	0.75	pSE[1]
No. of rats	4	4	4	4	4	
Initial wt (g)	158.9	155.0	164.1	162.6	159.4	6.91
Body weight[1,3,4] change (g)	-9.9	1.1	1.2	6.2	6.3	1.48
Daily feed[2,3] intake (g)	8.5	9.5	10.2	10.3	10.4	0.43
Total N[3,5] retained (mg)	67.0	189.8	259.2	290.3	199.3	60.65

[1] pSE=pooled standard error (19df) from the analysis of variance.
[2] The linear effect of arginine was significant ($P<0.0001$).
[3] The quadratic effect of arginine was significant ($P<0.04$).
[4] The arginine requirement was estimated to be 0.20±0.03%. The regression equation for the fitted broken line was: body weight change = $4.56+(-72.80)x(0.198-X)$, $R^2=0.76$; where X is % dietary arginine and (0.198-X) is defined as 0 at levels of arginine greater than 0.198%.
[5] The arginine requirement was estimated to be 0.22±0.12%. The regression equation for the fitted broken line was: nitrogen retention = $249.58+(-818.55)x(0.223-X)$, $R^2=0.77$; where X is % dietary arginine and (0.223-X) is defined as 0 at levels of arginine greater than 0.223%.

Table 3

Reproductive performance of primigravid rats fed varied levels of dietary arginine (Experiment 2)

Variable	Dietary Arginine (%)					pSE[1]
	0.0	0.15	0.35	0.55	0.75	
No. of rats	4	4	4	4	4	
Initial body weight (g)	203.1	202.4	189.1	183.8	198.1	17.14
Body weight[2,3] change (g)	-17.3	-3.0	11.3	22.6	18.3	9.07
No. of pups	9	7	9	7	10	2.80
Weight per pup (g)	4.3	4.5	4.5	4.9	4.6	0.42
Daily feed[2] intake (g)	8.2	10.6	13.2	13.5	13.5	0.98
Total N[2,4] retained (mg)	65.3	243.8	270.3	445.8	458.5	134.36

[1] pSE=pooled standard error (19 df) from the analysis of variance.
[2] The linear effect of arginine was significant ($P<0.01$).
[3] The arginine requirement was estimated to be 0.48±0.11%. The regression equation for the fitted broken line was: body weight change = 20.41+(-75.09)x(0.48-X), R^2=0.99; where X is % dietary arginine and (0.48-X) is defined as 0 at levels of arginine greater than 0.48%.
[4] The arginine requirement was estimated to be 0.61±0.21%. The regression equation for the fitted broken line was: nitrogen retention = 458.50+(-529.09)x(0.62-X), R^2=0.88; where X is % dietary arginine and (0.62-X) is defined as 0 at levels of arginine greater than 0.62%.

Table 4

Assessment of mammary gland infection status

	No. of rats	Micro-organism	RNA (mg)	DNA (mg)
Expt. 2:				
Treatments:				
0% Arg	3	Negative	31	9.2
	1	*Escherichia coli*	24	8.8
0.15% Arg	4	Negative	32	8.7
0.35% Arg	4	Negative	41	11.2
0.55% arg	3	Negative	36	9.4
	1	*Proteus mirabilis*	34	9.2
0.75% Arg	4	Negative	43	11.3
Expt. 3:				
Treatments:				
0.15% Arg	9	Negative	37	10.2
	3	*Citrobacter freundii*	40	11.7
			34	9.5
			24	9.5
0.60% Arg	8	Negative	53	12.3
	1	*Citrobacter freundii*	51	11.0
Expt. 4:				
Treatments:				
0% arg	6	Negative	22	11.1
	1	*Citrobacter freundii*	26	10.9
Pair-fed	4	Negative	46	16.8
Control	8	Negative	65	15.8
Arg-free (early Gest)	6	Negative	51	16.5
	1	*Citrobacter freundii*	46	17.6
8 g feed (early Gest)	5	Negative	69	16.4
	1	*Escherichia coli*	68	18.1

Table 5

Effect of arginine on mammary gland development in primigravid rats (Experiment 2)

Variable	Dietary Arginine (%) 0.0	0.15	0.35	0.55	0.75	pSE[1]
No of rats	4	4	4	4	4	
Total wet MG[2,3] weight (g)	4.56	5.87	7.08	6.52	7.61	1.24
MG weight/100 g[3] body weight (g)	2.47	2.95	3.64	3.15	3.51	0.58
Total DNA in MG (mg)	9.10	8.65	11.24	9.32	11.33	1.89
Total RNA in[3] MG (mg)	29.3	31.6	41.1	35.6	42.7	8.42
RNA/DNA	3.22	3.68	3.64	3.79	3.77	0.42
ODC[4] activity[5] (nmol CO_2 h^{-1} g MG^{-1} protein)	137.9	352.3	156.2	454.8	718.9	297.43
SAMDC[6] activity (nmol $CO_2 \cdot h^{-1} \cdot g$ MG $protein^{-1}$)	2.33	4.94	5.12	3.59	4.68	2.18
Arginase activity (umol $N \cdot 2\ min^{-1} \cdot g$ MG $protein^{-1}$)	81.3	70.4	59.3	72.5	84.3	24.5

[1] pSE=pooled standard error (19df) from the analysis of variance.
[2] MG=mammary gland.
[3] The linear effect of arginine was significant ($P<0.03$).
[4] ODC=ornithine decarboxylase.
[5] The linear effect of arginine was significant ($P<0.02$).
[6] SAMDC=S-adenosyl-L-methionine decarboxylase.

gland RNA, but not DNA increased with increasing dietary arginine levels. Ornithine decarboxylase activity responded positively and significantly ($P<0.02$) to dietary arginine; but activities of SAMDC and arginase were unaffected.

Arginine consumption was highly and significantly ($P<0.02$) correlated with mammary gland weight as a percent of body weight, ODC activity and mammary gland RNA content; but was not correlated with gland DNA content or the RNA:DNA ratio (Table 6). ODC activity was significantly ($P<0.03$) correlated with mammary gland weight as a percent of body weight and mammary nucleic acid concentrations. However, neither SAMDC nor arginase activity was correlated with mammary gland weight or mammary nucleic acid concentrations.

Experiment 3:

Rats fed an arginine-deficient diet ate less feed ($P<0.001$) and lost body weight ($P<0.001$) during gestation. However, number of pups born and their birth weight were not affected by dietary arginine concentration (Table 7). Four out of 21 rats tested positive for bacterial growth (Table 4). Mammary gland nucleic acid concentrations were not affected by presence of bacterial growth.

Absolute mammary gland weight, and mammary gland weight expressed as a percent of total body weight were depressed significantly by feeding the arginine-deficient diet. Total mammary gland DNA and RNA contents were reduced significantly in the arginine-deficient diet; however, the ratio of RNA to DNA was not affected by dietary

Table 6

Correlations between mammary gland variables measured in rats fed varied levels of dietary arginine during gestation (Experiment 2)[1]

	Arginine consumed (g)	MG[2] (% body weight)	RNA (mg)	DNA (mg)	RNA/ DNA	ODC[2] activity (nmol $CO_2 \cdot h^{-1} \cdot g$ MG Protein^{-1})	SAMDC[2] activity (umol N^{-1} 2 min^{-1} $\cdot g$ MG protein^{-1})	Arginase
Arg cons.	1							
MG	0.52[3]	1						
RNA	0.52[3]	0.84[4]	1					
DNA	0.41	0.77[4]	0.89[4]	1				
RNA/DNA	0.40	0.42	0.54[3]	0.11	1			
ODC	0.56[3]	0.56[3]	0.61[3]	0.50[5]	0.41	1		
SAMDC	0.21	0.14	0.07	0.10	0.39	0.05	1	
Arginase	0.08	0.12	0.04	0.08	0.26	0.34	0.11	1

[1] Twenty observations.
[2] MG=mammary gland; ODC=ornithine decarboxylase; SAMDC=S-adenosyl-L-methionine decarboxylase.
[3] Significant ($P<0.02$).
[4] Significant ($P<0.0001$).
[5] Significant ($P<0.03$).

Table 7

Effect of dietary arginine on reproductive performance and mammary gland development in primigravid rats (experiment 3)

Variable	Dietary Arg (%)		pSE[1]
	0.15	0.60	
No. of rats	12	9	
Initial weight (g)	193.8	181.1	
Body weight change (g)[2]	-10.5	11.5	7.01
Daily feed intake (g)[2]	10.2	12.3	0.83
No. of pups	9.0	10.0	2.91
Weight per pup (g)	4.1	4.5	0.41
Total wet MG[4] weight (g)[2]	5.89	7.19	0.76
MG weight per 100 g Body weight (g)[2]	3.04	3.97	0.38
Total DNA in MG (mg)[2]	9.02	12.03	1.74
Total RNA in MG (mg)[3]	36.9	53.3	10.73
RNA:DNA	4.11	4.32	0.84
ODC[5] activity (nmol $CO_2 \cdot h^{-1} \cdot$ g MG protein^{-1})	245.8	254.6	219.10
Arginase activity (umol N$\cdot$2 min$^{-1}\cdot$ g MG protein^{-1})	68.2	75.9	19.61

[1] pSE=pooled standard error (20df) from the analysis of variance.
[2] Significant ($P<0.001$).
[3] Significant ($P<.003$).
[4] MG=mammary gland.
[5] ODC=ornithine decarcoxylase.

argininеconcentration. Neither ODC, nor arginase activity was affected by dietary arginine concentration.

Arginine consumption was correlated significantly with mammary gland weight as a percent of body weight and mammary nucleic acid concentrations but was not correlated with ODC or arginase activity (Table 8). In contrast to experiment 1, ODC activity was not significantly correlated with mammary gland weight or mammary nucleic acid concentrations.

Experiment 4:

Litter size and weight were not affected by either feed or arginine restriction. However, arginine or feed restricted rats underwent net weight loss during the gestation period (Table 9). Three out of 32 rats tested positive for bacterial growth (Table 4). Mammary gland nucleic acid concentrations were not affected by presence of bacterial growth.

Total mammary gland weight and mammary gland weight expressed as a percent of total body weight were depressed significantly by pair feeding the control diet, and further depressed by ad libitum feeding of arginine-free diet (Table 10). However, restriction of either food intake or arginine intake during early gestation did not adversly affect mammary gland growth. Total mammary gland DNA and RNA concentrations of the pair-fed control group were comparable to controls, and were significantly higher than rats fed arginine-free diets. Moreover, mammary gland nucleic acid contents were not affected by feeding the arginine-free diet

Table 8

Correlations between mammary gland variables measured in rats fed varied levels of dietary arginine during gestation (Experiment 3)[1]

	Arginine consumed (g)	MG[2] (% body weight)	RNA (mg)	DNA (mg)	RNA/DNA	ODC[2] ---activity--- (nmol $CO_2 \cdot h^{-1} \cdot g\ MG^{-1}$ protein)	Arginase (umol $N \cdot 2\ min^{-1} \cdot g\ MG^{-1}$ protein)
Arg cons.	1						
MG	0.81[3]	1					
RNA	0.64[4]	0.73[3]	1				
DNA	0.69[3]	0.75[3]	0.82[3]	1			
RNA/DNA	0.19	0.27	0.63[4]	0.07	1		
ODC	0.03	0.04	0.21	0.02	0.39	1	
Arginase	0.18	0.17	0.07	0.03	0.15	0.22	1

[1] Twenty one observations.
[2] MG=mammary gland; ODC=ornithine decarboxylase.
[3] Significant ($P<0.001$).
[4] Significant ($P<0.002$).

Table 9

Reproductive performance of primigravid rats fed different levels of arginine during early and late pregnancy (Experiment 4)

Variable	Treatments[1]					
	1	2	3	4	5	pSE[2]
No. of rats	7	4	8	7	6	
Initial weight (g)	187.5	199.5	168.7	192.6	195.2	17.51
Body weight[3,4] change (g)	-11.6	-10.4	14.8	-1.0	-2.7	7.04
No. of pups	8	10	9	10	7	2.65
Weight per pup (g)	4.7	5.0	5.1	5.1	5.4	0.58
Daily feed intake (g)	10.0	10.0	11.8	10.1	10.1	0.97
Total Arg intake (g)	0.00	1.03	1.44	0.99	1.03	0.12

[1] 1) fed arginine-free diet ad libitum; 2) fed an amount of the 0.60% arginine control diet equal to the amount consumed the previous day by rats fed the arginine-free diet; 3) fed 0.60% arginine ad libitum (control); 4) fed arginine-free diet from d 6 to d 14 then control diet through parturition; and 5) fed 8 g of the control diet per day from d 6 to d 14 then ad libitum through parturition.

[2] pSE=pooled standard error (31df) from the analysis of variance.

[3] Treatment 2 vs treatment 3 ($P<0.0001$).

[4] Treatment 2 vs treatment 4 and 5 ($P<0.05$).

[5] Treatment 1 vs treatment 3 ($P<0.002$).

Table 10

Mammary gland development of rats fed different levels of arginine during early and late pregnancy (Experiment 4)

	Treatments[1]					
Variable	1	2	3	4	5	pSE[2]
No. of rats	7	4	8	7	6	
Total wet MG[3,4,6] weight (g)	3.84	4.96	5.51	5.66	6.28	0.86
MG weight/100[4] g body weight (g)	2.12	2.76	3.26	2.97	3.25	0.47
Total RNA in[4,8] MG (mg)	20.9	46.3	65.3	49.9	69.1	16.07
Total DNA in[4] MG (mg)	10.01	16.86	15.80	18.13	16.67	4.21
RNA:DNA[5,8]	2.21	2.90	4.26	2.81	4.12	0.97
ODC[7] activity[8,9] (nmol $CO_2 \cdot h^{-1} \cdot g$ MG protein^{-1})	276±278	395±283	326±157	297±225	1736±2421	
Arginase activity[4] (umol N·2 $min^{-1} \cdot g$ MG protein^{-1})	59.4	83.8	79.5	73.8	73.4	16.68

[1] 1) fed arginine-free diet ad libitum; 2) fed an amount of the 0.60% arginine control diet equal to the amount consumed the previous day by rats fed the arginine-free diet; 3) fed 0.60% arginine ad libitum (control); 4) fed arginine-free diet from d 6 to d 14 then control diet through parturition; and 5) fed 8 g of the control diet per day from d 6 to d 14 then ad libitum through parturition.
[2] pSE=pooled standard error (31df) from the analysis of variance.
[3] MG=mammary gland.
[4] Treatment 1 vs treatment 2 ($P<0.05$).
[5] Treatment 2 vs treatment 3 ($P<0.03$).
[6] Treatment 2 vs treatment 4 and 5 ($P<0.05$).
[7] ODC=Ornithine decarboxylase.
[8] Treatment 4 vs treatment 5 ($P<0.03$).
[9] Means±Sd. Because variances were heterogeneous, the ANOV was conducted on log transformed data.

during early gestation. Furthermore, RNA, RNA:DNA and ODC activity of feed restricted rats during early gestation were significantly higher than arginine restricted rats during early gestation. The ODC activity of feed restricted rats during early gestation was five-fold higher than other treatments. Arginase activity was depressed significantly in rats fed the arginine-free diet.

Total arginine intake was correlated significantly with mammary gland weight as a percent of body weight, mammary nucleic acid concentrations, RNA:DNA ratio and arginase activity; but was not correlated with ODC activity (Table 11). However, ODC activity was correlated significantly with RNA.

Table 11

Correlations between mammary gland variables measured in rats fed varied levels of dietary arginine during gestation (Experiment 4)[1]

	Arginine consumed (g)	MG[2] (% body weight)	RNA (mg)	DNA (mg)	RNA/DNA	ODC[2] activity (nmol CO_2 $\cdot h^{-1} \cdot g\ MG^{-1}$ protein)	Arginase activity (umol $N \cdot 2\ min^{-1} \cdot g\ MG^{-1}$ protein)
Arg cons.	1						
MG	0.70[3]	1					
RNA	0.73[3]	0.60[3]	1				
DNA	0.46[4]	0.37[5]	0.61[3]	1			
RNA/DNA	0.60[3]	0.49[4]	0.77[3]	0.01	1		
ODC	0.12	0.27	0.44[5]	0.19	0.30	1	
Arginase	0.47[4]	0.11	0.10	0.06	0.21	0.03	1

[1] Thirty two observations.
[2] MG=mammary gland; ODC=ornithine decarboxylase.
[3] Significant ($P<0.001$).
[4] Significant ($P<0.01$).
[5] Significant ($P<0.05$).

CHAPTER V

DISCUSSION

In experiments 1 and 2, both mature gravid and nongravid rats fed arginine-free diets did not undergo negative nitrogen balance. This is in agreement with Burroughs et al. (1940) who suggested that arginine was not essential for the maintenance of nitrogen equilibrium. However, Milner and Visek (1978a) found that urinary urea, citric acid, and orotic acid concentrations increased during gestation when a 12% casein basal diet was fed. Addition of 1% dietary arginine reduced citric acid excretion. Furthermore, arginine supplementation reduced orotic acid excretion during late gestation and early lactation which indicated that the rate of endogenous arginine synthesis failed to meet the arginine requirement during gestation and lactation (Milner and Visek, 1978a). This is in agreement with Pau and Milner (1981) who reported that urinary excretion of orotic acid and citric acid increased significantly when gravid rats were fed an arginine deficient diet. In the current study, addition of as little as 0.15% dietary arginine resulted in a 3-fold and 4-fold increase in nitrogen retention for nongravid and gravid rats, respectively. Kari et al. (1981) reported that increasing dietary arginine from zero to 0.75% reduced significantly the percentage of nitrogen excreted as urea

nitrogen.

In experiment 2, nongravid rats lost body weight when arginine-free diets were fed. However, as little as 0.15% arginine in the diet of nongravid rats prevented weight loss even though daily feed intake increased by 1 g compared to feed intake for rats fed the arginine-free diet. This indicates that endogenous synthesis of arginine was sufficient to maintain positive nitrogen balance but not sufficient for optimum growth. In contrast, Walf and Corley (1939) suggested that endogenously synthesized arginine met the maintenance requirement for adult rats.

Pau and Milner (1981) reported that rats lost weight throughout gestation when arginine deficient diets were fed. In the current study, gravid rats lost weight when arginine free or deficient diets were fed. Weight loss for gravid rats was twice that of nongravid rats. Gravid rats needed more than 0.15% arginine in the diet to prevent weight loss, which suggests that the arginine requirement for weight gain during pregnancy is greater than that for maintanence in the nongravid mature rats. Zartarian et al. (1980) also found that weight gain was impaired severely in gravid rats receiving a low protein diet compared with nongravid rats.

Feed consumption generally increases about 50% during gestation to maintain fetal growth (Cripps and Williams, 1975). Based upon current data, the arginine requirement for nongravid rats was 0.21%. The daily feed intake of nongravid rats was about 10 g which resulted in 2.1 mg of

arginine consumed daily. The arginine requirement for gravid rats was estimated to be 0.55%. Gravid rats consumed approximately 13.5 g of feed or an estimated 7.75 mg of arginine daily. Feed intake for gestating rats increased only 35%, whereas the daily arginine requirement increased by 300%. Simply increasing feed consumption was not sufficient to meet the gravid rat's requirement. Furthermore, maternal body weight gain in experiment 2 increased 100% as the arginine concentration in the diet increased from 0.35% to 0.55%. However, feed intake remained unchanged. This suggests that maternal body weight gain was the result of increased arginine consumption rather than increased feed consumption. This is in agreement with Sampson et al.(1986) who indicated that increasing intake of a diet deficient in protein quality and quantity will not necessarily improve lactation performance; however, improving protein quality or quantity with or without increasing feed intake will markedly improve lactation performance.

During pregnancy, nutrients are utilized by tissues involved in maintenance, maternal tissue growth, the developing fetus, and the mammary gland (Bauman and Currie, 1980). Rattary et al. (1974) reported that during pregnancy, when the conceptus is growing rapidly and making maximal demands on the dam, mobilization of maternal body reserves may occur to meet the demands of fetal growth. The number of pups per litter and their average weight were not

affected in rats fed a high protein (12% casein) or low protein (7.5% casein) diet during pregnancy (Zartarian et al., 1980).

Pau and Milner (1981), however, found that consumption of an arginine-free diet by the dam during gestation resulted in lower birth weight and higher perinatal mortality. In the current study, the number of pups born and their weight were not affected by dietary arginine concentration, which indicates that pregnancy can apparently be maintained by dependence on maternal tissue stores of amino acids for growth of the fetus. Hence, the high priority accorded the demands of the fetus may reduce severely the nutrient supply to the mammary gland.

Grigor et al. (1987) found that rats fed restricted amounts of protein or total diet had lower mammary gland mass and milk yield than did rats fed a control diet ad libitum. Rosso et al. (1981) reported that feed restriction from day 5 of gestation markedly reduced mammary gland weight compared to control, ad libitum fed animals. Affected glands also had a significant reduction in DNA, RNA and protein content. Thus, restriction of protein intake during pregnancy affects mammary gland development and protein synthesis, presumably by altering both cell number and rate of synthesis per cell.

Pau and Milner (1981) observed that consumption of an arginine deficient diet during gestation depressed significantly total mammary gland weight. The present study

indicated that restriction of arginine did not affect the number of pups born or the birth weight, but definitely affected mammary gland development, since the mammary gland weight or mammary gland as a percent of body weight was depressed significantly in the absence of dietary arginine in all three experiments. The depression in mammary gland weight was associated with significantly depressed mammary gland RNA and DNA content. This is in agreement with Pau and Milner (1982) who found that consumption of an arginine deficient diet during gestation depressed significantly total mammary gland nucleic acid contents, but the ratio of RNA : DNA was not affected by dietary arginine concentration. Results of the current study demonstrated that RNA:DNA ratio was not altered by dietary arginine in experiments 2 and 3 but was depressed significantly in experiment 4. The finding that increases in mammary gland mass and nucleic acid content could not be attributed to the increase in feed intake was demonstrated in experiment 4. Rats which were pair-fed the control diet had higher mammary gland mass and higher mammary nucleic acid contents than rats fed arginine-free diets even though feed restriction by pair-feeding resulted in depression in body weight. This is in agreement with Pau and Milner (1982) who indicated that depression in mammary gland nucleic acid content could not be attributed to reduced feed intake alone. In their study, rats pair-fed an amount of the control diet consumed the previous day by rats fed the arginine deficient diet did not

show depressed gland nucleic acid content. These results suggest that feeding an arginine-free diet to gravid rats markedly impairs mammary gland development by decreasing mammary gland mass and mammary gland nucleic acid contents.

Hawrylewicz et al. (1989) found that, at 7 wk of age, the ODC values for mammary epithelial cells from rats fed a high protein diet were significantly greater than that of rats fed a normal protein diet. These results indicate that increases in dietary protein concentration may stimulate RNA and protein biosynthesis in mammary epithelial cells of the developing mammary gland, since ODC activity has been implicated as an early response of cellular proliferation (Russell and McVicker, 1972). In experiment 2, arginine level significantly affected ODC activity. Results from this experiment substantiated the proposed hypothesis that arginine's role in mammary gland development appears to be related to its key role in supplying substrate for polyamine synthesis which is necessary for DNA and RNA synthesis for mammary gland growth. This is supported by Oka and Perry (1974a) who demonstrated that when mammary explants were cultured in medium in which the concentration of arginine was reduced from 4 mM to 0.2 mM, synthesis of spermidine and α-lactalbumin was inhibited. However, in experiments 3 and 4, an arginine effect on ODC activity was not observed. In experiments 3 and 4, even though an arginine treatment effect on ODC activity was not observed, there was a high correlation between RNA concentration and ODC activity.

Thus, it appears that although current evidence supports a key role of ODC in mammary gland development, and suggests that arginine has a substantial effect on mammary gland development, it does not appear that the effect of arginine is mediated directly via ODC. The effect of arginine on mammary gland development could be mediated via proline synthesis (Yip and Knox 1972), polyamine synthesis (Russell and Synder 1968), or via certain mammogenic or lactogenic hormones. Intravenous infusion of arginine has been shown to increase plasma prolactin (McAtee and Trenkle, 1971), growth hormone and insulin (Davis, 1972). Key lactogenic hormones, insulin, prolactin and glucocorticoid, promote increased levels of ODC and arginase (Oka and Perry, 1974c).

Results of the current studies indicated that when arginine-free diets were fed, both gravid and nongravid rats remained in positive nitrogen balance, but lost body weight. Neither number of pups born nor birth weight was affected by dietary arginine concentration. Mammary gland weight was depressed significantly in the absence of dietary arginine, and the depression in mammary gland weight was associated with significantly depressed mammary RNA and DNA concentrations. Morever, the RNA:DNA ratio was not affected by dietary arginine. Feed consumption of gestating rats increased only 35% whereas the dietary arginine requirement increased by 300%. Dietary arginine level did not consistently affect ODC activity; however, there was a high

correlation between ODC and RNA concentration.

Hence, dietary arginine was required for optimal mammary gland development during gestation. It appears that the effect of arginine was not mediated directly via ODC.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Attempts were made in this study to determine the effect and role of arginine in rat mammary gland development during gestation. The arginine requirement for nongravid and gravid mature rats based on nitrogen retention and body weight gain were also estimated.

Experiments were conducted to obtain information regarding effects of dietary arginine on nitrogen retention, body weight gain, mammary gland mass, mammary gland nucleic acid content, and enzymes involed in polyamine biosynthesis.

Mature gravid and nongravid rats fed arginine-free diets remained in positive nitrogen balance. When arginine-free diets were fed, both gravid and nongravid rats lost weight. However, as little as 0.15% dietary arginine prevented weight loss and increased nitrogen retention nearly 3-fold. The number of pups born and pup birth weight were not affected by dietary arginine.

Mammary gland weight or mammary gland weight as a percent of total body weight was depressed significantly in the absence of dietary arginine. The depression in mammary gland weight was associated with significantly depressed mammary RNA and DNA contents. However, the RNA:DNA ratio was not affected by dietary arginine. The increase in mammary gland mass and mammary gland nucleic acid contents

was not attributed to increased in feed intake, since feed intake for gestating rats increased only 35% while arginine increased by 300%. In addition, rats pair-fed the 0.60% arginine control diet had higher mammary gland mass and higher mammary nucleic acid contents than rats fed arginine-free diets even though feed restriction by pair-feeding did result in depression in body weight and mammary gland weight. It appears that simply increasing feed consumption was not sufficient to meet the rat's requirement.

Arginine level affected significantly ODC activity in experiment 2. The effect of arginine on ODC activity was not repeatable in the other experiments. However, there was a high correlation between RNA and ODC activity in the current study. Although our evidence supports a key role of ODC in mammary gland development, and suggests that arginine has a substantial effect on mammary gland development, it appears that the effect of arginine is not directly mediated via ODC. S-adenosyl-L-methionine decarboxylase and arginase activities were not affected by dietary arginine concentration.

Restricting arginine or feed intake during the early part of gestation followed by excess arginine or feed intake during late gestation did result in compensatory growth, and mammary gland mass and mammary gland nucleic acid concentrations were higher than in rats fed an arginine-free diet and were comparable to control rats. The arginine requirement for the nongravid rat, based upon nitrogen

retention and body weight gain was estimated to be 0.21%. The arginine requirement for gravid rats was estimated to be 0.55% which is in agreement with the NRC recommendation of 0.60%.

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VITA

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