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HORMONAL REGULATION OF
ALPHA-LACTALBUMIN SECRETION FROM
BOVINE MAMMARY TISSUE CULTURED
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HORMONAL REGULATION OF ALPHA-LACTALBUMIN SECRETION FROM BOVINE MAMMARY TISSUE CULTURED IN VITRO

By

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ABSTRACT

HORMONAL REGULATION OF ALPHA-LACTALBUMIN SECRETION FROM BOVINE MAMMARY TISSUE CULTURED IN VITRO

By

Gordon Timothy Goodman

Mammary tissue from nonlactating, multiparous cows 1 to 8 weeks prepartum was cultured in Medium 199 containing 5 µg insulin and .65 ng triiodothyronine per ml medium for 8, 24, 48 and 72 h. Explants were exposed to various combinations and doses of prolactin, growth hormone, estradiol-17β, cortisol and/or progesterone. Concentrations of α-lactalbumin in media and tissue homogenates were measured by radioimmunoassay or sodium dodecyl sulfate (SDS) gel electrophoresis as an index of lactogenesis.

Increasing concentrations of prolactin (25 to 100 ng/ml medium) progressively increased secretion of α-lactalbumin from bovine mammary tissue into media. Concentrations of α-lactalbumin in homogenates of mammary explants exposed to 100 ng prolactin, however, were not significantly different from α-lactalbumin concentrations in control explants. Thus, changes in α-lactalbumin concentrations in media throughout the duration of experiment reflected intracellular synthesis and release of α-lactalbumin from the mammary explants. De novo

synthesis and secretion of α-lactalbumin from mammary tissue in response to prolactin was confirmed in studies in which explants were exposed to medium containing ^{3}H-leucine for 3 h. Explants exposed to ^{3}H-leucine plus prolactin for 3 h incorporated approximately 2, 11, and 18 times more radioactivity at 21-24, 45-48, and 69-72 h after initiation of culture, respectively, than mammary tissue cultured in the absence of prolactin.

Cortisol in the absence of prolactin had no effect on α-lactalbumin secretion from bovine mammary explants. However, addition of cortisol into medium containing prolactin increased α-lactalbumin secretion approximately 2.4-fold over explants receiving prolactin alone, and 6.7-fold over explants receiving cortisol alone or control explants.

Addition of progesterone into media increased secretion of α-lactalbumin from mammary tissue approximately 2-fold over control explants. Addition of 10, 100, or 1000 ng per ml progesterone into media containing prolactin progressively decreased prolactin-induced α-lactalbumin secretion from 2.44 to 1.20, .75 and .54 ng/ml medium/mg tissue/h, respectively, 48 h after initiation of culture.

Estradiol-17β significantly increased secretion of α-lactalbumin from mammary explants over control explants. Moreover, addition of 10, 100, or 1000 ng of estradiol-17β

into the media synergized with prolactin to increase secretion of α-lactalbumin approximately 1.05, 1.31, and 1.63 fold, respectively, over explants receiving prolactin alone. Addition of progesterone dramatically reduced this synergistic effect between estradiol and prolactin.

Growth hormone at 10 or 100 ng/ml medium had no effect on α-lactalbumin secretion from mammary tissue. However, in the presence of prolactin, 100 or 1000 ng growth hormone increased α-lactalbumin secretion over that observed with prolactin alone.

I conclude that prolactin is the limiting hormone to lactogenesis in the cow, and that cortisol and estradiol-17β potentiate prolactin's lactogenic effects.

I dedicate this dissertation to my wife, Nancy Jean Goodman. Through her I can see beauty and excellence in a life filled with mediocrity, because of her I can pursue truth and knowledge, and beside her I can walk with security and love in my heart and mind.

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I would like to thank my wife, Nancy, for reinforcing my ideals and love for mankind, and for her support during turbulent times. To my fellow graduate students I am appreciative for encouraging me when I felt down-trodden, and humbling me when I was overconfident. And lastly to my parents I will be forever grateful for their love and instilling in me a lust for life and learning.

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INTRODUCTION

Virtually every hormone in the body has a direct or indirect effect upon mammary gland growth and lactation. The hormonal events associated with pregnancy and parturition are important signals for the onset of lactation, or lactogenesis, as well as the magnitude of the subsequent lactation. Learning how to manipulate the factors that regulate lactogenesis potentially could increase efficiency of milk production.

Increasing milk production could be accomplished via increasing numbers of secretory cells, increasing the potential or capacity of each cell to secrete milk, or increasing the functional life span of each cell. Understanding the role that each hormone plays provides clues for the manipulation of milk production.

The overall objective of these experiments was to study hormonal control of lactogenesis in cattle. This was accomplished by subjecting mammary tissue isolated from pregnant, nonlactating cows to various concentrations and combinations of various pituitary, ovarian and adrenal cortical hormones *in vitro*. Concentrations of these hormones have been demonstrated to either rise or fall in serum of cattle approaching parturition, and have been

implicated in the onset of lactation. However, the role of each of these hormones on the initiation of lactation in cattle is unclear. These studies were designed to evaluate the role of individual hormones on secretion of α-lactalbumin from bovine mammary tissue cultured in vitro. Appearance of α-lactalbumin is a biochemical index of the onset of lactation, and quantities of α-lactalbumin are an index of the magnitude of lactation.

REVIEW OF LITERATURE

Definition of Lactogenesis

Lactogenesis may be defined as initiation of milk secretion. Hartmann (1973) conveniently separated lactogenesis into two phases. The first phase is characterized by limited secretion of milk components in late pregnancy. During this phase the mammary secretory cells gradually acquire cellular organelles and enzymes necessary to synthesize and secrete milk proteins, carbohydrates and fatty acids. The second phase of lactogenesis is characterized by secretion of copious quantities of milk immediately prior to onset of parturition. The actual development of mammary secretory tissue during each of these phases is controlled by a hormonal mileau. Since so many hormones change in serum of the mother as parturition approaches, it is difficult to determine precisely which biochemical or cytological event occurring within the mammary cell is the result of each change in hormone concentration. It appears, at least superficially, that each of the cellular events occurring within the mammary secretory cell are interrelated and often regulated by the same hormones. It has not yet been possible to ascribe

precisely a role of each hormone to each event in the cytological and biochemical differentiation of the mammary secretory cell.

Cytological differentiation of the bovine mammary secretory cell includes: 1) increased adherence of ribosomes to the endoplasmic reticulum, 2) hypertrophy of the rough endoplasmic reticulum, 3) polarization of the endoplasmic reticulum and nucleus to the serosal portion of the secretory cell, 4) appearance of enlarged Golgi vesicles containing milk protein micelles, 5) increased ratio of cytoplasmic area to nucleoplasm, and 6) increased number and size of milk fat droplets (Heald, 1974).

Biochemical differentiation of the mammary secretory cell closely follows cytological differentiation. Enzymatic activity in mammary tissue rapidly increases as the animal approaches parturition. Baldwin and Milligan (1966) showed that many enzymes such as citrate cleavage enzyme, UDPGal-4-epimerase, phosphoglucomutase and glucose-6-phosphate dehydrogenase each increase over five fold in mammary tissue of rats between 6 days prepartum and 3 days post-partum. Mellenberger et al. (1973) demonstrated that bovine mammary tissue increases its capacity to synthesize fatty acids approximately 20-fold from 30 days prepartum to 40 days postpartum. They showed that other enzymes such as lactose synthetase, phosphoglucomutase and UDP-glucose-4-epimerase also increase during lactogenesis.

Therefore, structural and biochemical differentiation prepartum allows mammary secretory cells to secrete copious quantities of milk upon parturition. Hormonal changes that occur during pregnancy and parturition govern differentiation and expression of the mammary secretory cell.

It is generally believed that elevated concentrations of progesterone in blood of pregnant animals tonically inhibit onset of lactation, whereas elevations of the 'pro-lactational' hormones such as prolactin and cortisol stimulate lactogenesis. The following review will describe the hormonal control of development of the mammary gland during pregnancy and parturition. Since large numbers of mammary secretory cells are needed before secretion of copious quantities of milk can take place, a brief review of mammary growth will also be presented.

Hormonal Control of Mammary Growth and Lactogenesis

Hormonal Control of Mammary Growth

Between onset of puberty and conception, growth that occurs within the mammary gland is predominantly ductular. Physiologically, ductular tissue is for the most part nonsecretory in nature but does give rise to alveolar secretory tissue. Minimal hormonal requirements for ductal growth are estrogen and either growth hormone

or prolactin (Gomez et al., 1936). Cole (1933) first noticed large increases in ductular development with onset of the first estrous cycle at puberty in the mouse and each subsequent estrous cycle was accompanied by another small increase in mammary growth. Sinha and Tucker (1969) showed that allometric growth of the mammary gland is initiated well in advance of the first estrous cycle. They also showed that mammary DNA is lowest at proestrus, increases during estrus, and is followed by a gradual decrease during metestrus and diestrus in the heifer. However, net increases in ductular growth only occurred during the first few cycles after puberty, then reached a plateau until conception.

During pregnancy considerable amounts of lobule-alveolar as well as ductular development of the mammary gland occur. Alveolar development is characterized by formation of secretory units (alveoli), composed of a single layer of secretory cells surrounding a lumen. Estrogen is predominantly associated with ductular growth and differentiation, whereas progesterone is primarily responsible for lobule-alveolar development (Trentin and Turner, 1948; Lyons, 1958). Full lobule-alveolar development requires progesterone, estrogen and prolactin (Lyons et al., 1943). Insulin, an adrenal glucocorticoid and growth hormone all enhance mammary growth (Jeulin-Bailly et al., 1973; Prop, 1960; Wood et al., 1975).

Hormonal Control of Lactogenesis

The following review presents evidence that presence of prolactin, estrogen, and cortisol, and absence of progesterone is necessary for the onset of secretion of copious quantities of milk.

Prolactin.--Prolactin concentrations increase in blood immediately prior to parturition in the dairy cow (Edgerton and Hafs, 1973; Ingalls et al., 1973), goat (Hart, 1972), and ewe (McNeilly, 1971). Inhibition of periparturient secretion of prolactin by ergot alkaloids depresses onset of lactation in goats (Hart, 1976), cows (Karg et al., 1972; Johke and Hodate, 1978), and humans (Rolland et al., 1978), but not in ewes (Kann et al., 1974).

Akers et al. (1980) found that dairy cows given ergocryptine alone secreted 11.4 kg milk/day less than normal control cows during the first 10 days of lactation. Rates of lactose synthesis were 40% lower in ergocryptine-treated cows than in control cows. Periparturient infusion of prolactin for 6 days into additional cows given ergocryptine restored milk yields to levels in untreated control cows. These investigators concluded that prolactin is necessary for initiation of lactation. However, suppression of the periparturient secretion of prolactin did not completely block initiation of lactation, i.e. cows

given ergocryptine still produced 18 kg of milk/day. This indicates that basal concentrations of prolactin (less than 8 ng/ml) are adequate for lactogenesis to occur, but the periparturient surge of prolactin may be necessary for normal milk yields in the subsequent lactation.

To show further that prolactin is necessary for the onset of lactation removal of the anterior pituitary was accomplished. Injection of anterior pituitary extracts into hypophysectomized ferrets (McPhail, 1935), dogs (Houssay, 1935), and guinea pigs (Gomez and Turner, 1936) initiated milk secretion. Lyons et al. (1943) found that prolactin injected into a main duct of pseudopregnant hypophysectomized rat mammary gland stimulated growth and secretion in that segment only. This indicated prolactin's direct effect upon mammary tissue. Depletion of serum prolactin via hypophysectomy in the cow has not been demonstrated.

Mammary tissue excised from pregnant mammals and cultured in vitro allows one to determine the effect of hormones uncomplicated by the rest of the body. Elias (1957) first demonstrated that prolactin is needed for lobule-alveolar development of adult mouse mammary tissue cultured in vitro.

The mechanism by which prolactin stimulates protein, carbohydrate and fatty acid synthesis is currently under intense investigation. It is known that prolactin

stimulates synthesis of casein in mouse (Feldman, 1961; Terry et al., 1975), rabbit (Houdebine et al., 1975), and rat (Rosen et al., 1975) mammary tissue. Within 30 min after exposure of rat mammary tissue to prolactin, increased quantities of casein gene are transcribed (Guyette et al., 1979), and within 1 h significant quantities of casein mRNA accumulates (Matusik and Rosen, 1978). Therefore prolactin's ability to stimulate casein synthesis in the differentiated mammary cell is rapid. And prolactin stimulates casein gene transcription as well as translation.

Prolactin stimulates synthesis of other milk constituents as well as casein. Rabbit mammary tissue exposed to prolactin in vitro stimulates milk fat synthesis (Forsyth et al., 1972), lactose synthesis (Delouis and Denamur, 1972), β-lactoglobulin and α-lactalbumin (Turkington et al., 1967) synthesis. Jones and Forsyth (1969) reported that prolactin stimulates synthesis of other enzymes in mouse mammary explants such as ATP-citrate lyase, malic enzyme, lactate dehydrogenase and UDP-glucose phosphorylase.

There have been relatively few investigations examining the hormonal control of lactogenesis utilizing ruminant mammary tissue in vitro. Jeulin-Bailly et al. (1973) cultured mammary explants from pregnant ewes and exposed them to various hormonal combinations. They

demonstrated that little or no alveolar development occurred when tissues were exposed for 10 days to insulin, cortisol, prolactin, growth hormone and ovarian steroids. However, if the explants were initially exposed to estrogen, progesterone, insulin and cortisol for the first 2 days of culture, lobule-alveolar differentiation occurred. Supplementation with prolactin and growth hormone after this time enhanced secretory activity for up to 10 days of culture. Collier *et al*. (1977) observed that mammary explants obtained from pregnant, nonlactating cows cultured in the presence of cortisol, insulin and prolactin synthesized fatty acids approximately 3 fold faster than explants exposed to insulin and cortisol. Moreover, this increased rate of fatty acid synthesis was greatest 48 h after addition of prolactin to culture media. However, 72 h after initiation of culture there was histological evidence of cellular involution. For example, large amounts of lipid and protein micelles were present in the expanded alveolar lumen and accumulated within the epithelial cells; and the endomembrane system was undergoing regression. From these studies it is evident that with time secretory tissue cultured *in vitro* loses its ability to synthesize and secrete milk components into the medium. Unfortunately, very few studies measure milk constituents secreted into medium from mammary tissue cultured *in vitro*. It is also apparent from these studies

that prolactin increases fatty acid synthesis in bovine mammary tissue cultured *in vitro*.

In order for prolactin to exert its effect, it must bind to specific receptors on the mammary tissue membranes. Turkington (1970) first established that a specific receptor for prolactin exists on mammary tissue. Djiane *et al*. (1977) reported that number and affinity of prolactin receptors in the rabbit mammary gland remain low throughout pregnancy, then increase approximately 500% just prior to parturition along with increased concentrations of prolactin in blood. Because changes in number of prolactin receptors parallel changes in concentrations of prolactin in blood, it has been suggested that prolactin regulates its own receptor numbers in the mammary gland. Indeed, Posner *et al*. (1975) confirmed that prolactin induces prolactin receptors in mammary and liver tissue.

To demonstrate that binding of prolactin to prolactin receptors is necessary for lactogenesis to occur, Shiu and Friesen (1976) developed guinea pig antisera to partially purified prolactin receptors derived from rabbit mammary tissue. They showed that this antisera did not bind prolactin but did inhibit prolactin-mediated stimulation of ^{3}H-leucine incorporation into casein in rabbit mammary gland explants maintained in organ culture. In a later study (Bohnet *et al*., 1978) prolactin receptor antisera was given to post-parturient female rats for

5 days beginning within 1 h after delivery. Milk yield (as measured by litter weight gain) of mothers given this antisera decreased significantly. These data also suggest that binding of prolactin to receptor sites is necessary for prolactin to exert its lactogenic effect.

Prolactin's ability to stimulate lactogenesis is dependent on the intrinsic ability of the mammary secretory cell to transmit information from the prolactin-receptor interaction. Nagasawa and Yanai (1978) determined concentrations of prolactin in blood and binding characteristics of prolactin on mammary tissue of genetically inferior and superior lactating mice. There were no significant differences in binding activities, number of receptor sites, association constants of prolactin receptor sites, or prolactin concentrations in plasma and pituitary between superior and inferior lactating mice. These results indicate that neither quantity or quality of receptor sites, nor blood or pituitary concentrations of prolactin necessarily reflect the ability of the mammary secretory cell to perform its physiological function in superior or inferior lactating mice.

In conclusion, prolactin stimulates synthesis of milk proteins within mature mammary epithelial cells _in vivo_ and _in vitro_ for all species studied. Prolactin's ability to stimulate secretion of milk constituents into medium, however, is not known. Prolactin's role in

lactogenesis in bovine mammary tissue has received little study. The use of ergocryptine to depress the periparturient surge of prolactin depresses milk yields in the cow, but ergocryptine neither obliterates basal concentrations of serum prolactin in the cow, nor does it completely abolish lactogenesis. Therefore, the extent to which prolactin affects lactogenesis still remains to be determined in the cow. Since surgical removal of the hypophysis is difficult in the cow, another alternative is to use an *in vitro* system to elucidate the role of prolactin in lactogenesis.

Culture systems used previously have been relatively unreliable in that relatively subjective evaluations (i.e. histological ratings) were used to quantitate the ability of prolactin to stimulate secretion. Also, rather large quantities of prolactin have been utilized in the culture system, and secretion of milk constituents into media were not recorded.

Progesterone.--Lactogenesis occurs as a result of the release of the mammary gland from inhibition by progesterone and stimulation of the gland by glucocorticoids and prolactin (Kuhn, 1977). In the rat (Chatterton *et al.*, 1975), rabbit (Hillard *et al.*, 1973), cow (Convey, 1974), and other mammalian species (Kuhn, 1977) progesterone concentrations remain elevated in serum throughout pregnancy

until 2 to 3 days prior to parturition when they decrease precipitously. In all mammalian species tested, progesterone is required for maintenance of pregnancy, and is therefore likely to be a part of the mechanism by which onset of lactation is restrained until parturition occurs (Kuhn, 1969; Bedford et al., 1972).

Many investigators have demonstrated that ovariectomy of rats (Liu and Davis, 1967; Davis et al., 1972), rabbits (Denamur and Delouis, 1972), ewes (Hartmann, Trevethan and Shelton, 1973), or cows (Shirley et al., 1973) during pregnancy initiates lactogenesis. Drummond-Robinson and Asdell (1926) removed the corpora lutea from pregnant goats which resulted in the initiation of lactation. These studies further demonstrated that the presence of progesterone tonically inhibits onset of lactation in pregnant mammals. Moreover, administration of exogenous progesterone into rats (Deis, 1968; Yokayama, Shinde and Ota, 1969), rabbits (Denamur and Delouis, 1972) or sheep that have been hormonally or naturally induced into lactation, can prevent the onset of that lactation. Injection of progesterone into pregnant rats before parturition depresses subsequent postpartum milk production (Herrenkohl, 1971; Herrenkohl and Lisk, 1973).

Concurrent with the decline of serum progesterone before parturition, mammary tissue acquires the enzymes necessary to secrete copious quantities of milk components.

Turkington and Hill (1969) demonstrated *in vitro*, that progesterone selectively prevented the induction of α-lactalbumin synthesis; α-lactalbumin being the rate-limiting enzyme for lactose synthesis. And as progesterone concentrations decrease in serum, α-lactalbumin concentrations increase in mammary tissue (Turkington and Hill, 1969; Palmiter, 1969). Thus, progesterone can inhibit lactogenesis by inhibiting enzymes necessary for milk production.

Addition of progesterone into media containing insulin and cortisol blocks the lactogenic effect (based on histological appearance of milk in the alveolar lumen) of prolactin in mammary organ cultures from dogs (Barnawell, 1967), mice (Turkington and Hill, 1969), rabbits (Delouis and Terqui, 1974), and cows (Nickerson *et al.*, 1978). However, since progesterone was used at only one dose in these studies it is not known whether increasing concentrations of prolactin can overcome the inhibitory effects of progesterone.

Progesterone inhibits the lactogenic effects of prolactin without affecting prolactin's mammotrophic properties. For example, Assairi *et al.* (1974) showed that concommitant injection of progesterone plus prolactin in the pseudopregnant rabbit inhibited prolactin-induced increases of lactose synthetase, polyribosome formation

and RNA/DNA ratios but did not affect the prolactin-induced increase in mammary DNA.

Progesterone may also inhibit lactogenesis by inhibiting prolactin's ability to induce the synthesis or unmasking of prolactin receptors. Djiane and Durand (1977) injected prolactin with and without concommitant administration of progesterone into pseudopregnant rabbits. Their results indicated that progesterone inhibits prolactin's ability to induce its own receptor. However, neither progesterone, estrogen, testosterone nor cortisol affect prolactin's ability to bind to its mammary receptors (Shiu and Friesen, 1974).

Receptor sites for progesterone decrease in number parallel to the concentrations of progesterone in serum. Haslam and Shyamala (1979) using R5020, a synthetic progestin, revealed that the levels of progestin receptors in the mouse mammary gland are inversely proportional to the secretory activity of the gland. Their results indicated that specific binding of [^{3}H]-R5020 is present in virgin and early pregnant mice, but binding decreases during pregnancy and relative to virgin mammary tissue, binding was virtually absent from days 2 to 15 postpartum. These data suggest that the ability of progesterone to bind and thus inhibit lactogenesis declines as pregnancy advances.

The effects of progesterone on bovine mammary tissue have not been clearly established since few studies utilize ruminant mammary tissue. In the cow, serum concentrations of progesterone are elevated until a week before parturition then decrease precipitously approximately 3 to 5 days prepartum (Smith et al., 1973), yet concentrations of various enzymes necessary for milk secretion have substantial activities 30 days prior to parturition (Mellenberger et al., 1973; Mellenberger et al., 1974). Therefore, it is possible that progesterone tonically inhibits secretion of copious quantities of milk, but does not inhibit enzyme activity and/or protein synthesis late in gestation in the cow.

In vitro studies to determine the effects of progesterone on milk secretion would allow answers to three main questions: 1) Can increasing concentrations of prolactin overcome the inhibitory effects of progesterone on milk secretion from bovine mammary tissue?; 2) Is progesterone capable of inhibiting secretion but not synthesis of milk components in ruminant mammary tissue late in gestation?; and 3) Can other 'prolactional' hormones in addition to prolactin override progesterone's inhibitory effect on lactogenesis? In vitro studies of these problems will avoid general systemic effects such as progesterone's inhibitory effect on prolactin secretion (Meites, 1963; Karg and Schams, 1974).

Estrogen.--It was originally thought that estrogens were inhibitory to the onset of lactation because their removal from circulation stimulated onset of lactation. Nelson (1934, 1936) first showed that estrogens cause mammary growth in guinea pigs, and as soon as the administration of estrogen is discontinued, milk secretion occurs. The mechanism by which estrogens tonically inhibited the onset of lactation in these experiments may be by inhibiting prolactin release from the pituitary (Folley and Malpress, 1948; Turner and Meites, 1941). Indeed, Karg and Schams (1974) observed that infusion of estradiol-17β into lactating cows reduced prolactin concentrations in blood, but upon termination of infusion serum prolactin concentrations increased 2 to 5 fold. Thus, estrogens administered in relatively large doses can indirectly inhibit lactogenesis by tonically inhibiting prolactin release from the pituitary.

It is now generally believed that estrogen is stimulatory to the onset of lactation for the following reasons. Concentrations of estrogens in blood gradually increase throughout gestation reaching a zenith just prior to parturition then decrease postpartum in the rat (Yoshinga et al., 1969), pig (Baldwin and Stabenfeldt, 1975; Molokwa and Wagner, 1973), ewe (Bedford et al., 1972; Robertson and Smeaton, 1973), and cow (Henricks et al., 1972; Smith et al., 1973).

When administered in doses comparable to concentrations seen in various physiological states, estrogen stimulates lactogenesis. Estrogen increases prolactin secretion from the pituitary in the rat (Niswender et al., 1969; Chen and Meites, 1970), and cow (Padmanabhan and Convey, 1979). Meites and Turner (Meites, 1959) theorized the primary factor triggering milk secretion is the estrogen-induced release of prolactin from the pituitary. And during pregnancy estrogen and progesterone act together as potent inhibitors of milk secretion; however at parturition, when concentrations of progesterone decrease, estrogen becomes dominant and stimulates the anterior pituitary to secrete prolactin.

Considering that cows have relatively long gestation periods coupled with the fact that time wasted with animals exhibiting reproductive disorders cost relatively large sums of money has led many investigators to attempt to induce lactation in non-pregnant cows. Meites (1961) and Turner et al. (1956) demonstrated that prolonged treatment (approximately 6 months) with estrogen and progesterone followed by estrogen alone for 2 weeks initiates lactation in non-pregnant heifers. It was thought that estrogen-progesterone combination maximized lobule-alveolar development, while the withdrawal of progesterone and injection of estrogen initiated lactation. Benson et al. (1955) confirmed this in ovariectomized goats. In a review

article Meites (1961) pointed out that a wide spectrum of estrogen-progesterone combinations will induce lactation in ruminants, but variation in milk production resulting from artificial induction of lactation continued to preclude its practical use. As of 1974 estrogen and progesterone administered even at the most optimal doses and times known resulted in successful lactation in only 60% of the treated animals, and produced only 70% of expected milk production (Smith et al., 1974). Collier et al. (1977) hypothesized that prolactin may be the rate-limiting component in cows which fail to lactate following estrogen-progesterone treatment. To test this hypothesis they injected reserpine (a tranquilizer that increases serum prolactin), estrogen and progesterone into non-lactating cows. They found that reserpine administration increased prolactin levels, as well as milk yields in these cows. However, lactational yields were still not equal to previous milk yields obtained from normal pregnancy-induced lactations. Application of further knowledge gained by experiments geared towards elucidating the role of estrogen, progesterone, and prolactin could potentially increase milk yields in animals artificially induced into lactation.

It is also known that estradiol-17β stimulates release of prostaglandin $F_{2\alpha}$ from the uterus (Challis et al., 1972). Prostaglandin $F_{2\alpha}$ is luteolytic in several species and will cause lactogenesis in rats (Deis, 1971).

Louis et al. (1973) showed that prostaglandin $F_{2\alpha}$ stimulates release of a number of hormones including prolactin from the bovine pituitary. Thus, estrogen can stimulate lactogenesis by decreasing progesterone secretion and by increasing prolactin secretion.

To demonstrate the necessity of estrogen for lactogenesis, Abdul-Karim et al. (1966) showed that administration of an estrogen antagonist, ethamethoxy-triphetol, for 3 weeks prior to parturition in ewes almost completely inhibited milk secretion, but had no effect on mammary development.

It is apparent that estrogen indirectly stimulates mammary secretory tissue via decreasing progesterone and increasing prolactin secretion. However, the effect of estrogen directly upon mammary tissue has received comparatively little attention. Bolander and Topper (1980) reported that mouse mammary explants cultured in the presence of estradiol-17β augments the ability of prolactin to stimulate casein and lactose synthesis. This ability of estrogen to augment prolactin's effects directly on the mammary cell may be via increasing prolactin receptor sites since Sheth et al. (1978) demonstrated that administration of estrogen to mice stimulates prolactin binding in the mammary glands.

Nickerson et al. (1978) exposed mammary explants from heifers hormonally induced into lactation with

estradiol-17β and progesterone to medium containing various hormones and 30% calf plasma. Their results indicated that tissues exposed to medium containing progesterone, estrogen and prolactin exhibited greater lipid and protein secretion (as indicated by histological analysis) than explants exposed to the same medium without estrogen and progesterone. These data suggest that presence of both estrogen and progestins enhance the ability of prolactin to stimulate lactogenesis. However, the fact that 30% calf plasma was added to the medium coupled with the fact that neither estradiol-17β nor progesterone was added alone to the media clouds interpretation of the role of estrogen and progesterone in lactogenesis in these studies.

Cortisol.--There is little doubt that the presence of cortisol is necessary for at least the initial phase of lactogenesis, the biochemical and morphological maturation of the mammary epithelial cell. However, cortisol's role in the secretion of copious quantities of milk is not as clear. Concentrations of cortisol in blood increase several-fold prior to parturition, then decline a few days after parturition in cows (Smith et al., 1973), and sows (Baldwin and Stabenfeldt, 1973). The rise in blood cortisol concentrations has been attributed to stress of

parturition and increased fetal adrenal activity (Zarrow et al., 1972; Oka et al., 1974).

Injection of cortisol into pregnant mice (Nandi and Bern, 1961), rabbits, rats (Talwalker et al., 1961), sheep (Delouis and Denamur, 1967), and heifers (Tucker and Meites, 1965) initiates lactation and premature abortion when relatively large doses are administered. Thus, in vivo it is difficult to determine whether cortisol directly affects mammary tissue to secrete milk, or indirectly via enhancement of other lactogenic hormones. Lyons et al. (1958) injected various hormonal combinations into hypophysectomized-ovariectomized-adrenalectomized female rats, and demonstrated that minimum requirements for milk secretion were prolactin and cortisol.

Elias (1957, 1959) demonstrated that insulin and cortisol inhibited regression of adult mouse mammary tissue cultured in vitro. Oka and Topper (1971) further demonstrated that cortisol stimulates accumulation of rough endoplasmic reticulum in mouse mammary epithelial cells. Cortisol also increases incorporation of free ribosomes into membrane bound ribosomes on the endoplasmic reticulum (Mayne et al., 1968; Green et al., 1971; Green and Topper, 1970). Therefore, presence of cortisol in vitro is necessary for at least the initial phase of lactogenesis which is associated with increasing protein synthesizing organelles in the mammary secretory cell.

Specific binding of cortisol by mammary tissue from a number of species during various physiological states have been observed. Paterson and Linzell (1971) demonstrated that mammary tissue uptake of cortisol increases 6-fold with the onset of lactation in the goat. Shyamala (1973) observed that mammary tissue from virgin and pregnant rats bound less cortisol than lactating mammary tissue. Gorewit and Tucker (1976) demonstrated that nonlactating, non-pregnant cows bound 336 molecules cortisol per mammary cell; 1-mo prepartum nonlactating cows bound 532 molecules cortisol per mammary cell; and 1263 molecules cortisol were bound per cell from lactating non-pregnant cows. Thus, the mammary cell binds progressively increasing concentrations of cortisol as the animal advances from pregnancy to lactation.

Tucker et al. (1971) demonstrated that progesterone reduces cortisol binding in bovine mammary cells cultured in vitro, but estradiol and testosterone are without effect. Progesterone reduces binding of cortisol in rat (Gardner and Witliff, 1973), mouse (Shyamala, 1973), vole (Turnell et al., 1974), and cow (Collier and Tucker, 1978) mammary tissue.

Cortisol potentiates prolactin's ability to stimulate milk secretion in pseudopregnant rabbits (Denamur, 1964; Delouis and Denamur, 1972). Devinoy et al. (1978) showed that the ability of cortisol to enhance prolactin's

ability to stimulate milk protein synthesis is via increasing the amount of mRNA translation, and that cortisol alone is relatively ineffective in stimulating milk protein synthesis.

Thus, cortisol is necessary for at least the initial phase of lactogenesis by preparing the mammary secretory cell with the cellular organelles necessary to secrete copious quantities of milk. However, it is not clear whether cortisol is necessary for the second phase of lactogenesis, the actual secretion of copious quantities of milk.

Insulin.--Concentrations of insulin are elevated in early stages of pregnancy then decrease throughout the duration of pregnancy in rats (Sutter-Dub et al., 1974), and cows (Koprowski and Tucker, 1973).

Insulin is necessary for maintenance of mammary tissue in vitro and in vivo (Rudland et al., 1977; Rillema, 1975; Oka and Perry, 1974) and is required for mammary tissue cultured in vitro to respond to lactogenic hormones (Lasfargues, 1957; Prop, 1960; Rivera and Bern, 1961; Voytovich and Topper, 1967). Insulin stimulates uptake of glucose which is essential for cellular metabolism (Mayne and Barry, 1970; Morretti and Abraham, 1966; Wang et al., 1972), nuclear protein phosphorylation (Turkington and Riddle, 1969), RNA polymerase activity (Turkington and

Ward, 1969), and RNA synthesis (Green and Topper, 1970) in mammary tissue. More recently, Linebaugh and Rillema (1978) showed that insulin enhances rate of uptake and incorporation of [^{3}H]-uridine into RNA, [^{3}H]-thymidine into DNA, and [^{3}H]-leucine into protein in mouse mammary epithelial cells. It is evident that insulin is necessary for maintenance and growth of mammary tissue both *in vivo* and *in vitro*.

For many years it was postulated that insulin stimulated differentiation and multiplication of mammary secretory cells since insulin increased DNA synthesis in mammary tissue cultured *in vitro* (El-Darwish and Rivera, 1970; Lockwood *et al.*, 1967; Prop *et al.*, 1965). These data led Stockdale and Topper (1966) to suggest that mammary epithelial cells must undergo an insulin dependent 'critical mitosis' which generates daughter cells capable of responding to lactogenic hormones. Vonderhaar and Topper (1974) later postulated that mammary cells from mature virgin animals have to progress through an insulin-dependent mitosis, while cells from mid-pregnant animals or primiparous animals do not. Thus, in order for mammary epithelial cells to synthesize and secrete milk proteins in response to lactogenic hormones, the cells must be at or through the G1-phase of their cell cycle, and to reach this phase insulin must be present. Vonderhaar *et al.* (1979) confirmed this hypothesis by demonstrating that

mature mammary cells obtained from virgin mice must traverse the G1-phase of the cell cycle to make casein and α-lactalbumin, but mammary cells obtained from primiparous mice are able to make these proteins without addition of insulin.

Recently, Devinoy et al. (1979) measured the capacity of mammary tissue from pseudopregnant rabbits to synthesize casein. Their results indicated that mammary tissue exposed to prolactin, in the absence of insulin and/or cortisol, stimulates casein synthesis. Although cortisol and/or insulin amplifies prolactin's ability to stimulate casein synthesis, neither cortisol nor insulin alone stimulate casein synthesis. These data suggest that neither insulin nor cortisol is lactogenic per se, but amplify the ability of prolactin to induce the onset of lactation.

Thus, insulin is neither lactogenic nor mitogenic in mouse or rabbit mammary tissue but does potentiate the ability of other hormones to stimulate mitosis and lactogenesis. Insulin is necessary for the maintenance, growth and secretion of mammary tissue both in vitro and in vivo.

Growth hormone.--Concentrations of growth hormone in serum remain low throughout pregnancy then dramatically increase on the day of parturition in cows (Ingalls et al., 1973; Johke and Hodate, 1977; Olsen et al., 1974),

humans (Grumbach et al., 1968), and ewes (Basset et al., 1970).

It has been suggested that the rise in serum growth hormone around parturition is due to stress of parturition and does not have a direct role in the initiation of lactation. However, growth hormone does not usually respond to stressful stimuli in cattle (Tucker, 1971; Johke, 1978). Shirley et al. (1973) demonstrated a marked increase in both growth hormone and prolactin when a cow's fetus was removed by caesarian section which further indicates that the elevated serum growth hormone is not due to the stress of labor. However, it is possible that the increase in serum growth hormone prior to parturition is due to estrogen. For example, Trenkle (1970) demonstrated that cattle consuming 10 mg diethylstilbestrol per day exhibited increased growth hormone concentrations in plasma. It has been suggested that the increased elevations of serum estrogen prior to parturition may stimulate growth hormone release from the pituitary of cattle approaching parturition (Hunter et al., 1970; Smith et al., 1972).

There is very little evidence that the increase in serum growth hormone at parturition is necessary for the onset of lactation. Lyons (1958) suggested that prolactin and cortisol are minimal hormonal requirements for initiation of lactation in mature rats, and growth hormone

augments this combination. Nandi (1959) showed that once lobule-alveolar formation was induced by treatment with estrogen, progesterone, cortisol, prolactin and growth hormone, milk secretion could be induced by injection of prolactin and cortisol alone, although in some strains of mice growth hormone could be substituted for prolactin.

Barnawell (1965) demonstrated that prolactin stimulated milk secretion in mammary tissue obtained from various species, and any apparent secretory effect of growth hormone could be accounted for by contamination of growth hormone preparations by prolactin. Therefore, in all species tested any apparent lactogenic effects of growth hormone could be accounted for by contamination with prolactin. It is well known that many growth hormone preparations have substantial cross-reactivities with prolactin preparations as seen by radioreceptor assay, radioimmunoassay (Handwerger and Sherwood, 1974), and bioassay (Forsyth, Folley and Chadwick, 1965).

The role of growth hormone in lactogenesis in cattle, however, may be different than in other species. In contrast to the ruminant, injection of growth hormone into lactating mice and rats has no galactopoietic effects as judged by litter weight gain (Cowie et al., 1957; Meites, 1957). Since growth hormone is galactopoietic in ruminants (Cotes et al., 1949), it is possible that the periparturient surge of growth hormone is lactogenic in

cattle but not in laboratory animals. And since no specific long-acting blocker to growth hormone release has been found, it would be necessary to test the effects of bovine growth hormone on milk secretion from bovine mammary tissue cultured *in vitro*.

Thyroid hormones.--Johke and Hodate (1978) reported that plasma concentrations of triiodothyronine, thyroxine or thyrotropin do not change throughout the duration of pregnancy or parturition in cows. Thyroidectomy between days 13 and 16 of pregnancy in rats did not affect subsequent parturition or lactation (Nelson and Tobin, 1937). These data suggest that thyroid hormones are not rate-limiting to the initiation of lactation.

Singh and Bern (1969) demonstrated that relatively low concentrations of thyroxine synergize with prolactin to stimulate lobule-alveolar development in mouse mammary tissue cultured *in vitro*. Vonderhaar (1975) reported that thyroid hormones enhance the ability of prolactin to stimulate α-lactalbumin activity as much as 5-fold in mouse mammary tissue cultured *in vitro*. Vonderhaar and Greco (1979) showed that decreased concentrations of thyroid hormones in serum or medium retards growth of ductular and alveolar tissue of mouse mammary gland *in vivo* and *in vitro*.

To summarize, thyroid hormones seem to be necessary for maximal development and maintenance of mammary tissue and regulation of milk protein synthesis in the mouse. However, it's role in bovine mammary tissue development and lactogenesis and *in vitro* remains to be elucidated.

Alpha-Lactalbumin: Physiological Importance of and Rationale for Use as an Indicator of Lactogenesis

Lactose synthetase is a two-component enzyme consisting of α-lactalbumin and galactosyl transferase (Brodbeck and Ebner, 1966). Galactosyl transferase catalyzes the transfer of galactose from UDP-galactose to N-acetylglucosamine to form N-acetylactosamine (Brew *et al.*, 1968). Galactosyl transferase is normally found in many tissues, and its purpose is to form and attach the carbohydrate moiety to glycoproteins (McGuire *et al.*, 1965). In the presence of α-lactalbumin, the substrate acceptor specificity of galactosyl transferase is modified to accept glucose instead of N-acetyl glucosamine (Brew, 1969). Therefore, α-lactalbumin permits the synthesis of lactose, a disacharride consisting of galactose plus glucose. For this reason α-lactalbumin has been designated a "specifier protein" (Brew *et al.*, 1968) that controls the rate of lactose synthesis.

In 1967, Brew and Campbell provided the first cytological evidence leading to the understanding of the

mechanism by which lactose is synthesized and secreted. During lactation, α-lactalbumin is synthesized on ribosomes associated with the rough endoplasmic reticulum. The α-lactalbumin is then transported into channels of the endoplasmic reticulum to regions of the membrane within the Golgi apparatus where galactosyl transferase is bound. Lactose is synthesized within the cisternae of the Golgi, then transported to and released from the cell surface into alveolar lumen, along with α-lactalbumin.

The biochemical and physiological significance of α-lactalbumin is three-fold: Firstly, continuous flux of α-lactalbumin through the mammary cell ensures that α-lactalbumin concentrations at any given time reflect rate of change of its synthesis since α-lactalbumin is not degraded by the cell like other enzymes (Brew, 1969). Secondly, rate of α-lactalbumin synthesis is proportional to lactose synthesis since α-lactalbumin is rate-limiting to lactose synthetase activity. Thirdly, α-lactalbumin concentrations increase in the mammary cell at a very low rate until just prior to parturition when quantities increase many fold (Brew *et al.*, 1968). Turkington *et al.* (1968) demonstrated that prolactin stimulates a 10-fold increase in galactosyl transferase and a 2-fold increase in α-lactalbumin in mouse mammary tissue cultured *in vitro*. In a later study Turkington and Hill (1969) demonstrated that progesterone selectively inhibits α-lactalbumin

formation but not galactosyl transferase in mouse mammary explants cultured *in vitro*.

Thus, α-lactalbumin can serve as a key indicator of lactogenesis because it's synthesis and activity is governed by hormonal controls. Prolactin stimulates and progesterone inhibits α-lactalbumin synthesis in the mouse. Alpha-lactalbumin is not degraded by the cell, and is naturally secreted into the alveolus by the mammary secretory cell. *In vitro*, α-lactalbumin can be measured in tissue and in media in response to various hormonal combinations. For these reasons, I chose to use α-lactalbumin as an index of lactogenesis.

MATERIALS AND METHODS

Rationale for Animal Model

Mammary tissue was obtained from pregnant (6 weeks before expected parturition), multiparous, non-lactating cows. During this stage of pregnancy, the mammary gland is exposed to relatively low concentrations of prolactin, cortisol and estradiol as compared with those concentrations that occur during the last few days before parturition. Thus, mammary tissue obtained from cows 6 weeks before parturition might be expected to be especially sensitive to various lactogenic hormones.

Rationale for Tissue Culture

Culturing whole mammary tissue, as opposed to dispersed cells, allows various tissue specific functions to be retained such as hormonal stimulation of milk secretion (Forsyth, 1971). Other advantages of this culture model include: relatively small quantities of tissue are necessary to perform an experiment, relatively short periods of time are necessary to obtain results, and the model permits study of effects of hormones directly on mammary tissue, uncomplicated by the rest of the body.

Tissue Sampling

Approximately 2.5 cm^3 of mammary tissue was removed surgically from the caudal aspect of a front mammary quarter while the cow was under general anaesthesia (Thiamylal, 0.2% intraveneously and halothane, 2.5-4.0%). Mammary tissue was blotted of excess blood and placed into ice cold, basal medium until tissue was sliced (approximately 20 min).

Tissue Culture Procedure

Mammary tissue trimmed of excess fat, connective and bloody tissue, was sliced into 1 to 2 mm slices using a Stadie-Riggs hand microtome, then diced into explants of 1 to 2 mm^3. Explants were rinsed several times in basal medium, then three or four explants (each 7 to 21 mg) were placed on a stainless steel grid (#3014 Organ Culture Grid, Falcon Plastics Co., Oxnard, CA). The center well of each culture dish (#3010 Organ Culture Dish, Falcon Plastics Co., Oxnard, CA) contained 1 ml culture medium, and the outer well contained a filter pad ring saturated with double distilled water to minimize tissue desiccation. The metal grid rested over the center well at the surface of the medium. Capillary movement of medium over tissue supplied nutrients and removed cellular by-products.

Basal medium for all cultures consisted of Medium 199 with Earles salts and L-Glutamine (Grand Island

Biological Company, Grand Island, NY). Sodium bicarbonate (2.2 g / L), sodium acetate (10 mM) and Hepes (15mM; Sigma Chemical Co., St. Louis, MO) were added to the basal medium. Antibiotics added to the basal medium included penicillin G (100 units / ml medium; Grand Island Biological Company, Grand Island, NY), sodium or potassium streptomycin sulfate (100 µg / ml medium; Grand Island Biological Company, Grand Island, NY) and amphotericin B (Fungizone, 2.5 µg / ml medium, Grand Island Biological Company, NY). Medium, antibiotics and buffers were combined, the pH adjusted to 7.35, then sterilized by filtration (Nalgene filter, 0.20 micron, Nalge Sybron Corp., Rochester, NY). All hormones were added to medium after filtration. Unless otherwise noted, basal medium also contained insulin, cortisol and triiodothyronine. Insulin (Sigma Chemical Co., St. Louis, MO) was dissolved in sterile 0.005 N HCl then added to basal media to a final concentration of 5 µg / ml medium. Cortisol (Sigma Chemical Co., St. Louis, MO) was initially dissolved in 10% ethanol, then added to culture media to a final concentration of 0.5 µg / ml medium. Triiodothyronine (L-triiodothyronine, Sigma Chemical Co., St. Louis, MO) was initially dissolved in basic (2.0 N NaOH) absolute ethanol, diluted with double distilled water, and finally added to medium to a final concentration of 0.65 pg / ml medium.

Depending upon the experiment, prolactin, growth hormone, estradiol and progesterone were added in various doses and combinations. Prolactin (NIH-B4, National Institutes of Health, Bethesda, MD) was dissolved in sterile 0.001 N NaOH and added to culture media to a final concentration of either 25, 50, 75 or 100 ng / ml medium. Growth hormone (NIH-B18; National Institutes of Health, Bethesda, MD) was dissolved in sterile 0.001 N NaOH and added to culture medium to a final concentration of 10, 100, or 1000 ng / ml medium. Estradiol-17β and progesterone (Sigma Chemical Co., St. Louis, MO) were dissolved separately in absolute ethanol and added to culture medium to a final concentration of 10, 100 or 1000 ng / ml medium. The final concentration of ethanol in the culture media never exceded 0.1%. Rivera (1964) demonstrated that ethanol concentrations below 0.5% in media have no deleterious effects on mammary tissue cultured *in vitro*.

In all aspects of the culture procedure, care was taken to maintain aseptic conditions. For the duration of incubation the culture dishes were maintained in an incubator at 37±2 C and gassed with 95% oxygen: 5% carbon dioxide mixture. All control and experimental media were added at the initiation of culture, and harvested and replaced 8, 24 and 48 h after 72 h of culture. Media were frozen at -4 C until assayed for α-lactalbumin.

At the termination of each experiment, unless otherwise noted, mammary explants were rinsed three times with Tris-sucrose buffer, blotted, weighed, then homogenized in 1 ml ice-cold Tris-salt buffer (see Appendix A for buffer solutions). Tissue was homogenized on ice using 10 strokes of a Ten Broeck Tissue Grinder (Corning 7726, Thomas Scientific, Philadelphia, PA). The homogenate was then centrifuged at 800 x g for 5 min and the supernatant fluid was frozen until assayed for α-lactalbumin by radioimmunoassay or sodium dodecyl sulfate (SDS)-gel electrophoresis.

Alpha-Lactalbumin Assay

Alpha-lactalbumin was quantified in medium and mammary tissue homogenate according to the method of Beck and Tucker (1977). Standard media samples were prepared by diluting α-lactalbumin (Sigma Chemical Co., St. Louis, MO) in Medium 199 with Earles Salts (Grand Island Biological Company, Grand Island, NY). Since the medium was harvested at unequal intervals (8, 24, 48 and 72 h after initiation of culture) concentrations of α-lactalbumin found in the experimental media were divided by tissue weight and time (in h) elapsed since the previous media change. Therefore, α-lactalbumin in medium was expressed as ng α-lactalbumin/ml medium/mg tissue/h. Concentrations of α-lactalbumin in mammary tissue homogenate were expressed as ng α-lactalbumin/mg tissue.

Specific Experimental Designs

Experiment 1: Effect of Prolactin on Secretion of α-Lactalbumin from Bovine Mammary Tissue Cultured In Vitro

Mammary explants from five cows were exposed to media containing 0, 25, 50, 75 or 100 ng prolactin/ml medium. Media were harvested and replaced at 8, 24 and 48 h after initiation of culture. Final harvest of media occurred at 72 h of culture and all media were assayed for α-lactalbumin by radioimmunoassay as described previously.

Experiment 2: Effect of Prolactin on α-Lactalbumin Concentrations in Bovine Mammary Tissue Cultured In Vitro

Mammary explants from 5 cows were exposed to media containing 0 or 100 ng prolactin/ml medium. Medium was harvested and replaced at 8, 24, 48 and 72 h after initiation of culture. Tissue explants of five dishes in each treatment were homogenized at either 8, 24, 48 or 72 h after initiation of culture. Homogenates and media were assayed for α-lactalbumin. Alpha-lactalbumin in medium was expressed as the total quantity of α-lactalbumin secreted into medium until the explant was homogenized.

Experiment 3: Effect of Prolactin on ^{3}H-Leucine Incorporation into α-Lactalbumin from Bovine Mammary Tissue Cultured In Vitro

Mammary tissues from two cows were diced into explants as outlined previously. Approximately 36 mg of mammary explants per dish were exposed to 1 ml medium containing 0 or 100 ng prolactin. Cultures were initiated at time 0 and medium harvested 8, 24, 48 and 72 h later. Five, 21, 45 or 69 h after initiation of culture, two dishes of tissues containing 0 ng prolactin/ml medium and two dishes containing 100 ng prolactin/ml medium were exposed to 1 ml of their respective treatment medium containing 0.1 m Ci [^{3}H]-leucine ([DL-4, 5-^{3}H (N)]-Leucine, New England Nuclear, Boston, MA). After 3 h, the medium was harvested and the tissue rinsed and homogenized as described previously. Also 5, 21, 45 or 69 h after initiation of culture, explants from two additional plates of tissue exposed to 0 or 100 ng prolactin/ml medium, but not exposed to ^{3}H-leucine, were rinsed, weighed and homogenized as described previously. Media and homogenates were frozen until assayed for α-lactalbumin by radioimmunoassay and SDS-gel electrophoresis.

SDS-gel electrophoresis was performed on radioactive medium and homogenates according to the method of Laemmli (1970) as outlined in Appendix B. Briefly, 65 µl of the

medium of homogenate samples were diluted with 15 µl of sample buffer and 2 µl α-lactalbumin standard (1 mg α-lactalbumin dissolved in 1 ml 0.005 N NaOH) to accentuate the α-lactalbumin band uptake of stain. The samples were boiled for 5 min, allowed to cool, then electrophoresed on a 3% stacking gel-12.5% separating gel at 2 mAmp/sample until the dye front entered the separating gel, then the amperage was increased to 4 mAmp/sample. Gels were stained with Coomassie blue as described by Fairbanks et al. (1971), scanned with a continuous recording Gilford 240 Spectrophotometer, then sliced into 2 mm slices. Each slice was counted in a scintillation spectrometer. Radioactivity in the α-lactalbumin band was expressed as counts per min/mg tissue to account for variance in weight of tissue exposed to radioactive leucine. One α-lactalbumin standard and one or two known protein standards were electrophoresed with every electrophoresis run. Alpha-lactalbumin standard consisted of 10 µl α-lactalbumin (1 mg/ml), 20 µl of sample buffer and 70 µl double distilled water. Known protein standard consisted of 10 µl bovine serum albumin (1.4 mg/ml), 10 µl ovalbumin (1 mg/ml) 20 µl prolactin (0.5 mg/ml), 10 µl growth hormone (1 mg/ml), 20 µl sample buffer and 30 µl double distilled water. Some protein standards also contained 10 µl α-lactalbumin (1 mg/ml) in which case only 20 µl of double distilled water was added.

Experiment 4: Effect of Cortisol on Secretion of α-Lactalbumin from Bovine Mammary Tissue Cultured In Vitro

Mammary explants from three cows were exposed to media containing cortisol (0.5 μg/ml medium), prolactin (100 ng/ml medium) or cortisol plus prolactin. Media were harvested and replaced 8, 24, 48 and 72 h after initiation of culture. Media were frozen at -4 C until assayed for α-lactalbumin.

Experiment 5: Effect of Progesterone on Secretion of α-Lactalbumin from Bovine Mammary Tissue Cultured In Vitro

Mammary explants from three cows were exposed to media containing 0, 10, 100 or 1000 ng progesterone in the absence of presence of 100 ng prolactin. Media were harvested, replaced, and assayed for α-lactalbumin at 8, 24, 48 and 72 h after initiation of culture.

Experiment 6: Effect of Estradiol-17β on Secretion of α-Lactalbumin from Bovine Mammary Tissue Cultured In Vitro

Mammary explants from three cows were exposed to media containing 0, 10, 100 or 1000 ng estradiol-17β in the absence or presence of 100 ng of prolactin. Media were harvested, replaced and assayed for α-lactalbumin at 8, 24, 48 and 72 h after initiation of culture.

Experiment 7: Interactions Among Estradiol-17β, Progesterone and Prolactin on Secretion of α-Lactalbumin from Bovine Mammary Tissue Cultured In Vitro

Mammary explants from three cows were exposed to media containing estradiol-17β (0, 100 ng/ml medium), progesterone (0, 100 ng/ml medium) and/or prolactin (0, 100 ng/ml medium) in a 2^3 factorial study. Media were harvested, replaced and assayed for α-lactalbumin at 8, 24, 48 and 72 h after initiation of culture.

Experiment 8: Effect of Growth Hormone on Secretion of α-Lactalbumin from Bovine Mammary Tissue Cultured In Vitro

Mammary explants from three cows were exposed to media containing 0, 10, 100 or 1000 ng growth hormone in the absence or presence of prolactin (100 ng/ml medium). Media were harvested, replaced and assayed for α-lactalbumin at 8, 24, 48 and 72 h after initiation of culture.

Statistics

All data were tested for heterogeneous variance using an F max test of variances (Gill, 1978a). In cases where heterogeneous variance existed, data were converted to logarithmic form before analysis of variance was performed. Data involving repeat measure over time (experiments 1, 2 and 3) were analyzed by split-plot

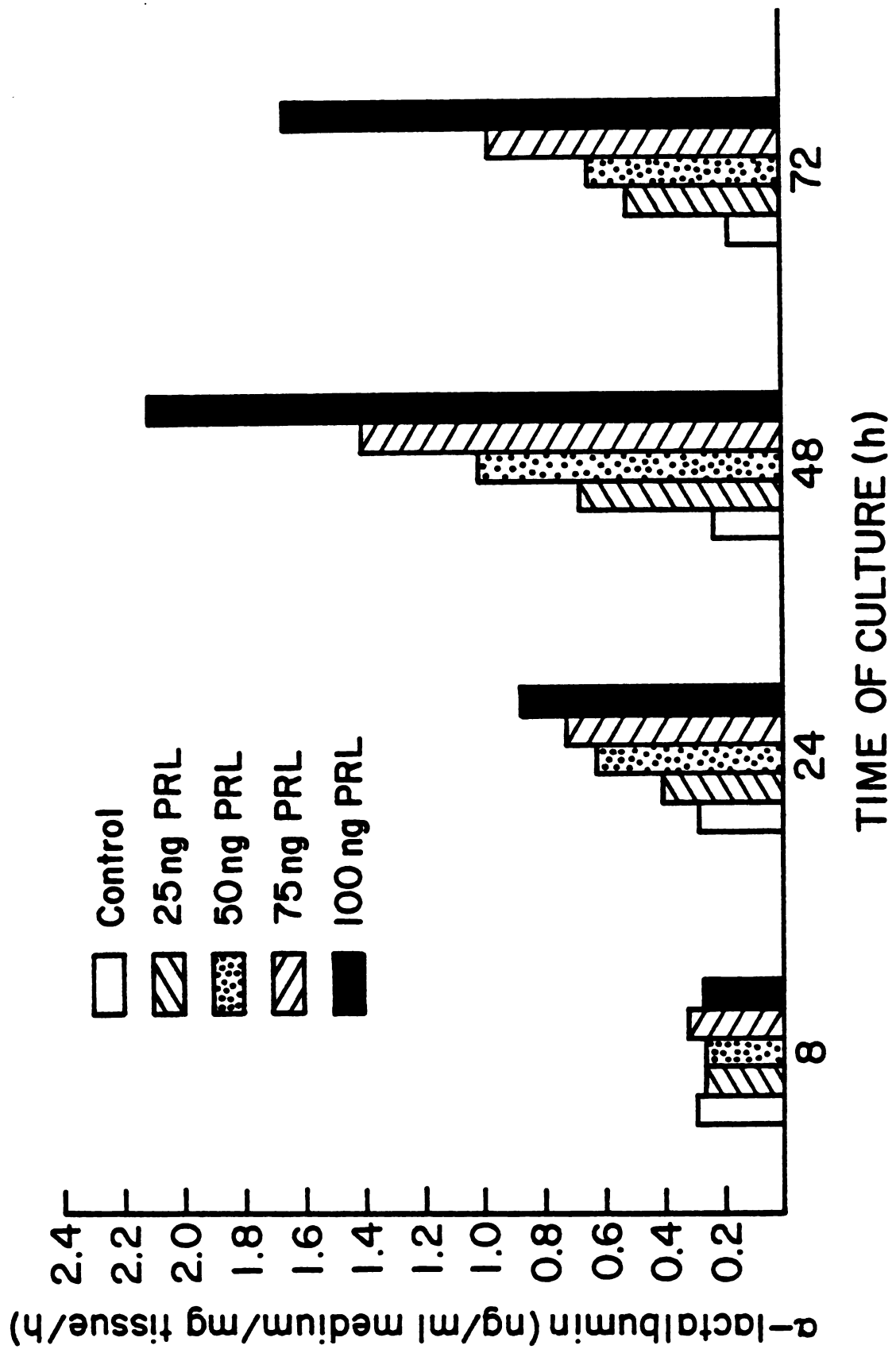
analysis of variance (Gill and Hafs, 1971). Specific comparisons of means were by orthogonal contrasts (experiment 4), by Bonferroni-t (experiments 5, 6 and 8) or Dunnetts-t test (experiments 1 and 2). Furthermore, the factorial experiment (experiment 7) was analyzed by Yates analysis of variance (Gill, 1978a, 1978b).

RESULTS

Effect of Prolactin on α-Lactalbumin Secretion into Media

Mean concentrations of α-lactalbumin secreted into media from mammary explants exposed to various concentrations of prolactin are illustrated in Figure 1. Eight h after initiation of culture, explants cultured in the absence of prolactin secreted .29 ng α-lactalbumin/ml medium/mg tissue/h. Alpha-lactalbumin secretion declined with time, such that 72 h after initiation of culture without prolactin explants secreted .18 ng α-lactalbumin/ml medium/mg tissue/h. This decline, however, was not statistically significant ($P>.05$). Prolactin did not affect α-lactalbumin production within 8 h after initiation of culture ($P>.05$). However, explants cultured in the presence of increasing concentrations of prolactin released increasing quantities of α-lactalbumin into medium 24, 48, and 72 h after initiation of culture ($P<.01$). Furthermore, secretion of α-lactalbumin into medium in response to 50, 75 and 100 ng prolactin was greater 48 h after initiation of culture ($P<.01$) than at 24 or 72 h of culture. Since mammary explants secreted greatest concentrations of α-lactalbumin in response to 100 ng prolactin/ml medium 48 h after initiation of culture, subsequent experiments

Figure 1.--Mean concentrations of α-lactalbumin in medium secreted from bovine mammary explants exposed to 0, 25, 50, 75 or 100 ng prolactin/ml medium for 8, 24, 48, or 72 h. Standard error of difference between means within the same culture period is .05. Standard error of differences between means among culture periods within treatment is .17.


α-lactalbumin (ng/ml medium/mg tissue/h)
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Control
25 ng PRL
50 ng PRL
75 ng PRL
100 ng PRL
8
24
48
72
TIME OF CULTURE (h)

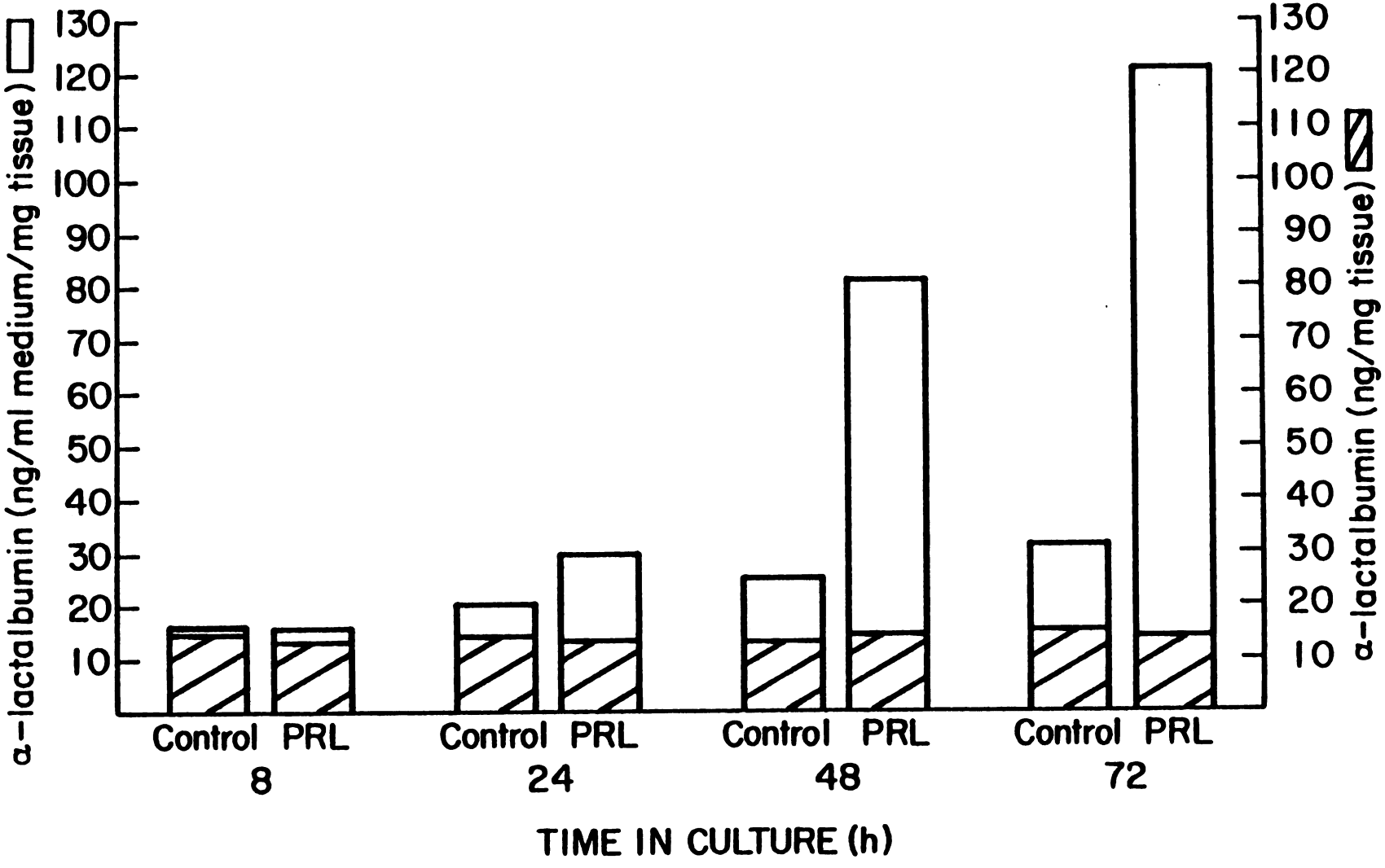
utilized prolactin at 100 ng/ml medium, and statistical analyses were focused on the 48 h period of culture, unless otherwise noted.

Effect of Prolactin on Concentrations of α-Lactalbumin in Tissue and Media

Concentrations of α-lactalbumin in tissues exposed to 0 or 100 ng prolactin/ml medium for 8, 24, 48, or 72 h of culture are expressed in Figure 2. Concentrations of α-lactalbumin in homogenates of mammary tissue exposed to either 0 or 100 ng prolactin/ml medium averaged 14 ng/mg tissue and these concentrations in tissue were not significantly different between treatments ($P>.05$) or over time of culture ($P>.05$).

However, rate of mammary secretion of α-lactalbumin into the media was dependent on duration of the experiment as well as presence or absence of prolactin. For example, explants exposed to 100 ng prolactin/ml medium secreted a total of 81 ng α-lactalbumin/ml tissue into the medium 48 h after initiation of culture and 120 ng α-lactalbumin/mg tissue into medium 72 h after initiation of culture. Explants cultured in the presence of prolactin secreted 3.2 and 3.8 times the quantity of α-lactalbumin into medium at 48 and 72 h after initiation of culture, respectively, than explants cultured in the absence of prolactin.

Figure 2.--Cumulative mean concentrations of α-lactalbumin in medium collected at 8, 24, 48 and 72 h after initiation of culture (□), and average concentrations of α-lactalbumin in homogenates of mammary tissue (▨) cultured in the absence or presence of 100 ng prolactin/ml medium for 8, 24, 48, or 72 h. Standard error of differences between means of two treatments at the same culture period is 8.8. Standard error of differences between means for the same treatment at different culture periods is 11.2.


α-lactalbumin (ng/ml medium/mg tissue)
α-lactalbumin (ng/mg tissue)
130
120
110
100
90
80
70
60
50
40
30
20
10
Control PRL
8
24
48
72
TIME IN CULTURE (h)

Prolactin Stimulation of Radioactive Leucine Incorporation into α-Lactalbumin

Figure 3 illustrates the mobility of various standard proteins separated via SDS-polyacrylamide gel electrophoresis. Relative mobility of each protein was calculated utilizing the equation:

$$\text{mobility} = \frac{\text{(distance of protein migration)}}{\text{(distance of dye migration)}} \times \frac{\text{(gel length before staining)}}{\text{(gel length after destaining)}}$$

Gel lengths were measured before and after staining because gels expand during the staining process (Cooper, 1977). As seen in Figure 3, the dye exclusion limit of this particular 12.5% gel was approximately 10,000 Daltons. Alpha-lactalbumin (MW 14,000) was clearly distinguishable from the growth hormone standard (MW 20,000) and the dye front (ca. 10,000 Daltons). Thus, we were able to add α-lactalbumin to all unknown media and tissue samples before electrophoresis in order to increase protein stain uptake without forfeiting accuracy. Increased stain uptake allows greater resolution of the band containing α-lactalbumin from other protein bands.

Figure 4 represents radioactivity in serial slices of two SDS-polyacryamide gels run with representative samples of medium from tissue exposed to 0 or 100 ng prolactin for 48 h and ^{3}H-leucine for 3 h. As seen in the upper panel of Figure 4, there was relatively no

Figure 3.--SDS-polyacrylamide gel electrophoretic profile of various standard proteins of known molecular weights and their relative mobility. Molecular weights of the standards are: bovine serum albumin (BSA), 63,000; ovalbumin (OVAL), 43,000; bovine prolactin (PRL), 22,000; bovine growth hormone (GH), 20,000; and bovine α-lactalbumin (α-LACT), 14,400.

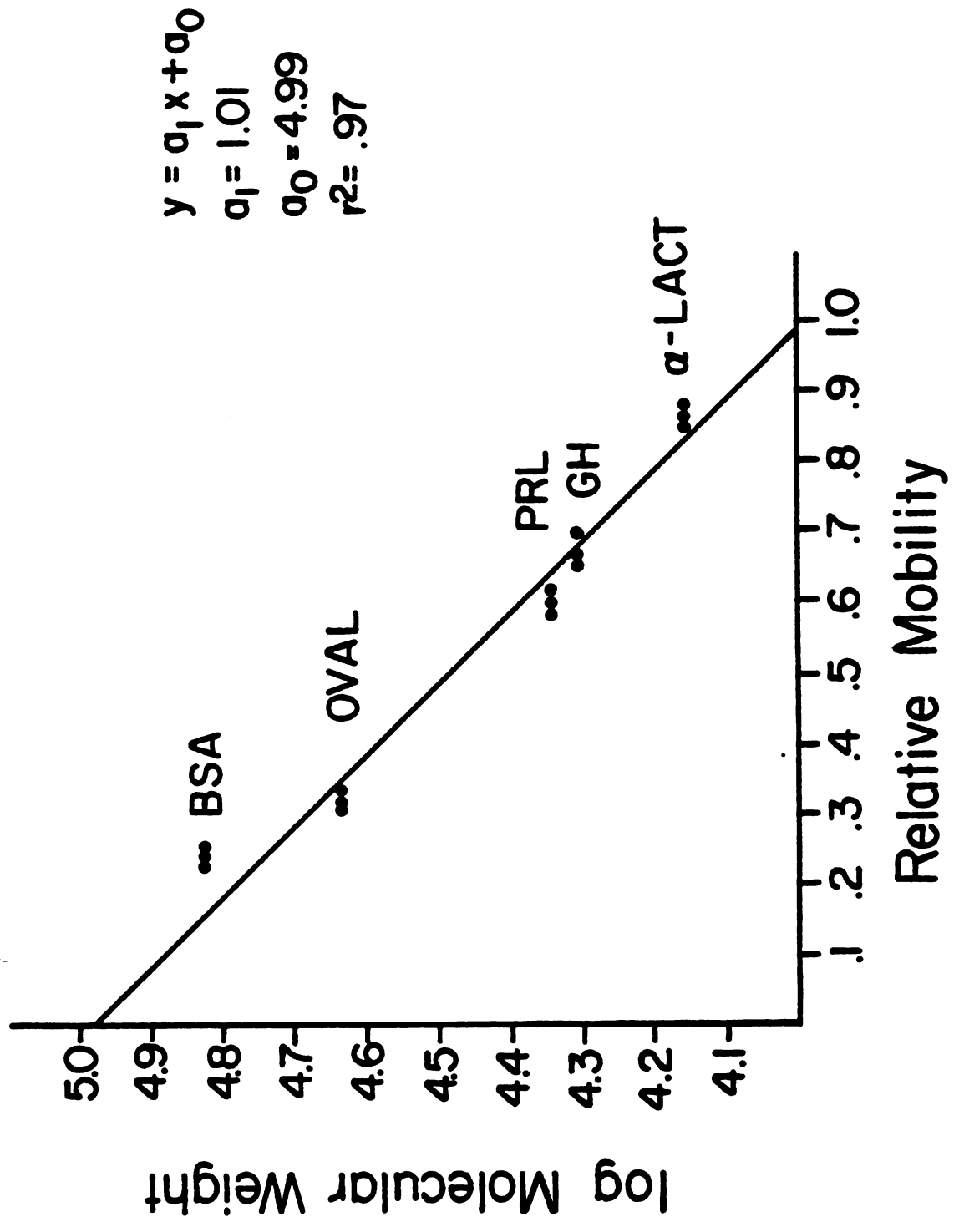
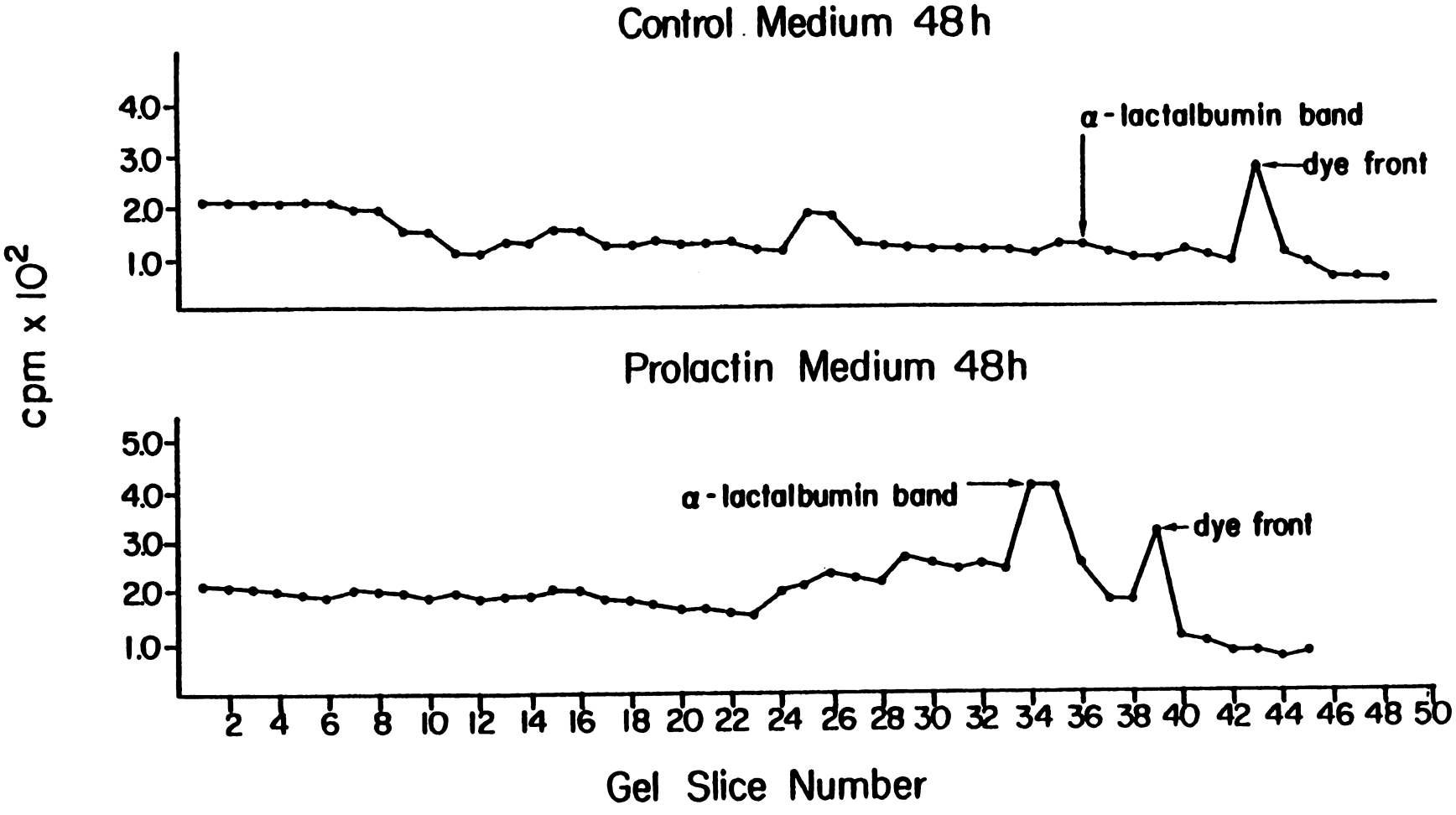

y = a1 x + a0
a1 = 1.01
a0 = 4.99
r2 = .97
BSA
OVAL
PRL
GH
α-LACT
log Molecular Weight
5.0
4.9
4.8
4.7
4.6
4.5
4.4
4.3
4.2
4.1
.1 .2 .3 .4 .5 .6 .7 .8 .9 1.0
Relative Mobility

Figure 4.--SDS-polyacrylamide gel electrophoretic profile of medium from mammary tissues exposed to 0 or 100 ng prolactin/ml medium for 48 h and ^{3}H-leucine between hours 45 and 48.


Control Medium 48h
α-lactalbumin band
dye front
4.0
3.0
2.0
1.0
cpm x 10^2
Prolactin Medium 48h
5.0
4.0
3.0
2.0
1.0
α-lactalbumin band
dye front
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Gel Slice Number

radioactivity in the corresponding α-lactalbumin band from medium of tissues cultured in the absence of prolactin. However, tissue cultured in the presence of prolactin secreted relatively large amounts of radioactive α-lactalbumin into the medium, as seen in the lower panel of Figure 4.

Electrophoretic gel profiles of homogenates of tissues exposed to 0 or 100 ng prolactin for 48 h and to ^{3}H-leucine between hours 45 and 48 is shown in Figure 5. Radioactive α-lactalbumin was found in tissues cultured in the absence as well as the presence of 100 ng prolactin. Tissues exposed to prolactin had larger amounts of ^{3}H-α-lactalbumin than tissues cultured in the absence of prolactin.

Mean rates of incorporation of ^{3}H-leucine into α-lactalbumin in tissues exposed to 0 or 100 ng prolactin/ml medium are summarized in Table 1. Explants exposed to prolactin incorporated approximately 2, 11, and 18 times more ($P<.01$) ^{3}H-leucine into α-lactalbumin at 21-24, 45-48 and 69-72 h after initiation of culture, respectively, than those explants cultured in the absence of prolactin. Furthermore, explants exposed to prolactin incorporated significantly greater quantities of ^{3}H-leucine into α-lactalbumin between 45-48 h than between 5-8, 21-24 or 69-72 h after initiation of culture ($P<.005$).

Figure 5.--SDS-polyacrylamide gel electrophoretic profile of homogenates of mammary tissue cultured in the presence or absence of 100 ng prolactin/ml medium for 48 h and ^{3}H-leucine between hours 45 and 48.

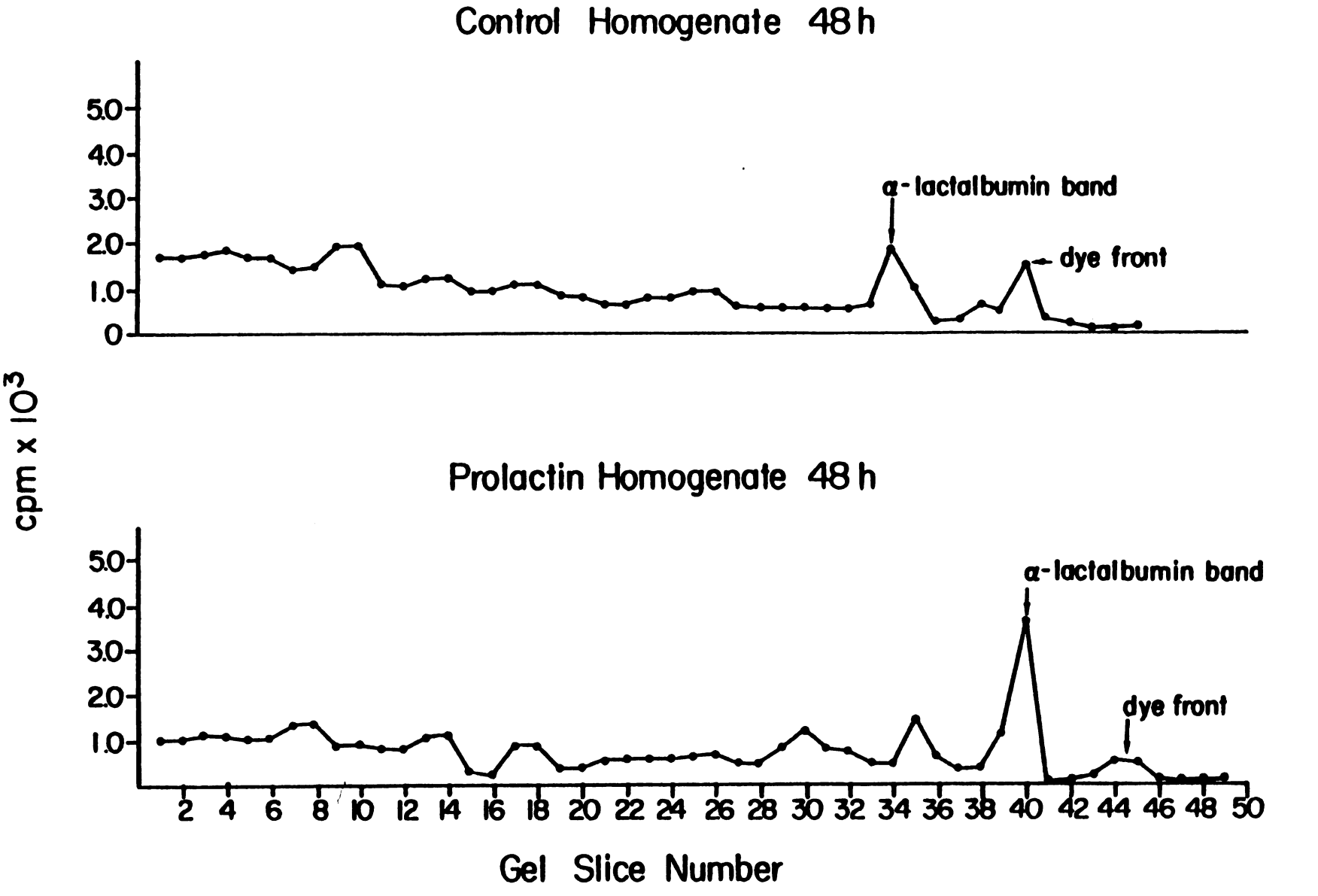

Control Homogenate 48 h
5.0
4.0
3.0
2.0
1.0
0
α-lactalbumin band
dye front
cpm x 10³
Prolactin Homogenate 48 h
5.0
4.0
3.0
2.0
1.0
α-lactalbumin band
dye front
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Gel Slice Number

Figure 5.--SDS-polyacrylamide gel electrophoretic profile of homogenates of mammary tissue cultured in the presence or absence of 100 ng prolactin/ml medium for 48 h and ^{3}H-leucine between hours 45 and 48.

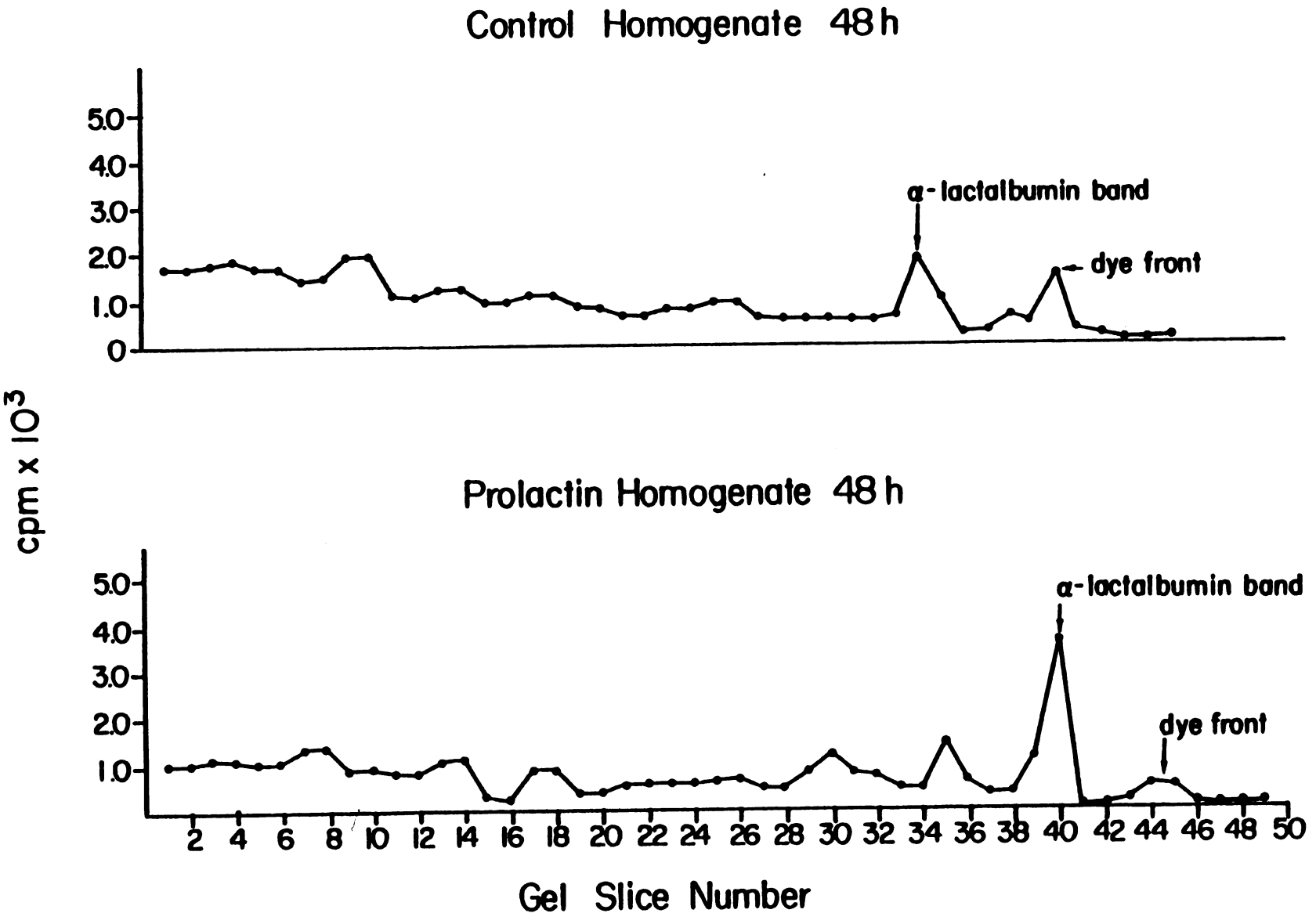

Control Homogenate 48 h
5.0
4.0
3.0
2.0
1.0
0
α-lactalbumin band
dye front
cpm x 10^3
Prolactin Homogenate 48 h
5.0
4.0
3.0
2.0
1.0
α-lactalbumin band
dye front
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Gel Slice Number

TABLE 1.--^{3}H-leucine incorporated into α-lactalbumin in medium and homogenate of mammary tissue cultured in the absence or presence of prolactin (100 ng/ml medium).

	Time of Culture (h)			
	5-8	21-24	45-48	69-72
Control homogenate (cpm/mg tissue/h)	113.3±32.6[a]	166.9±24.2[b]	84.3±27.6[b]	29.4±11.1[b,d]
Medium (cpm/mg tissue/h)	7.9±4.4	6.6±2.9[b]	13.8±5.2[b]	7.9±3.1[b]
Prolactin homogenate (cpm/mg tissue/h)	196.3±46.0	325.5±51.8	908.6±110.3[c]	539.9±73.9[d]
Medium (cpm/mg tissue/h)	6.3±3.0	19.6±6.8	34.1±6.0[c]	42.0±9.0

[a]Rates of ^{3}H-leucine incorporated into α-lactalbumin were calculated from quantities of radioactivity in SDS-polyacrylamide gel slice corresponding to the endogenous and external α-lactalbumin band. Values are means ± S.E.M.

[b]Less ($P<.01$) than values from explants exposed to prolactin within the same time period.

[c]Greater ($P<.01$) than values from explants exposed to the same treatment during the previous time period.

[d]Less ($P<.01$) than explants exposed to the same treatment during the previous period.

Effect of Cortisol on α-Lactalbumin Secretion

Mean concentrations of α-lactalbumin secreted into medium from mammary tissue cultured in .5 μg cortisol and/or 100 ng prolactin for 48 h are illustrated in Figure 6. Explants cultured in the presence of cortisol secreted an average of .27 ng α-lactalbumin/ml medium/mg tissue/h which was not significantly different ($P>.05$) from secretion of explants cultured in the absence of cortisol. Prolactin, without cortisol, significantly increased α-lactalbumin secretion from .24 to .77 ng/ml medium/mg tissue/h ($P<.001$). The combination of cortisol and prolactin increased α-lactalbumin secretion to 1.82 ng/ml medium/mg tissue/h ($P<.001$).

Effect of Progesterone on α-Lactalbumin Secretion

Addition of increasing concentrations of progesterone to medium with or without prolactin is shown in Figure 7. Prolactin alone in media stimulated explants to secrete an average of 2.44 ng α-lactalbumin/ml medium/mg tissue/h. Explants exposed to 10, 100, or 1000 ng progesterone/ml medium secreted .40, .32 and .36 ng α-lactalbumin/ml medium/mg tissue/h, respectively. These concentrations were significantly greater ($P<.05$) than control levels of .16 ng α-lactalbumin/ml medium/mg tissue/h. Addition of 10, 100 or 1000 ng of progesterone to media containing prolactin significantly decreased

Figure 6.--Mean concentrations of α-lactalbumin in medium secreted from bovine mammary tissue cultured *in vitro* after exposure to cortisol (0 or .5 μg/ml medium) and prolactin (0 or 100 ng/ml medium) for 48 h. Standard error of differences between means of treatments is .03.

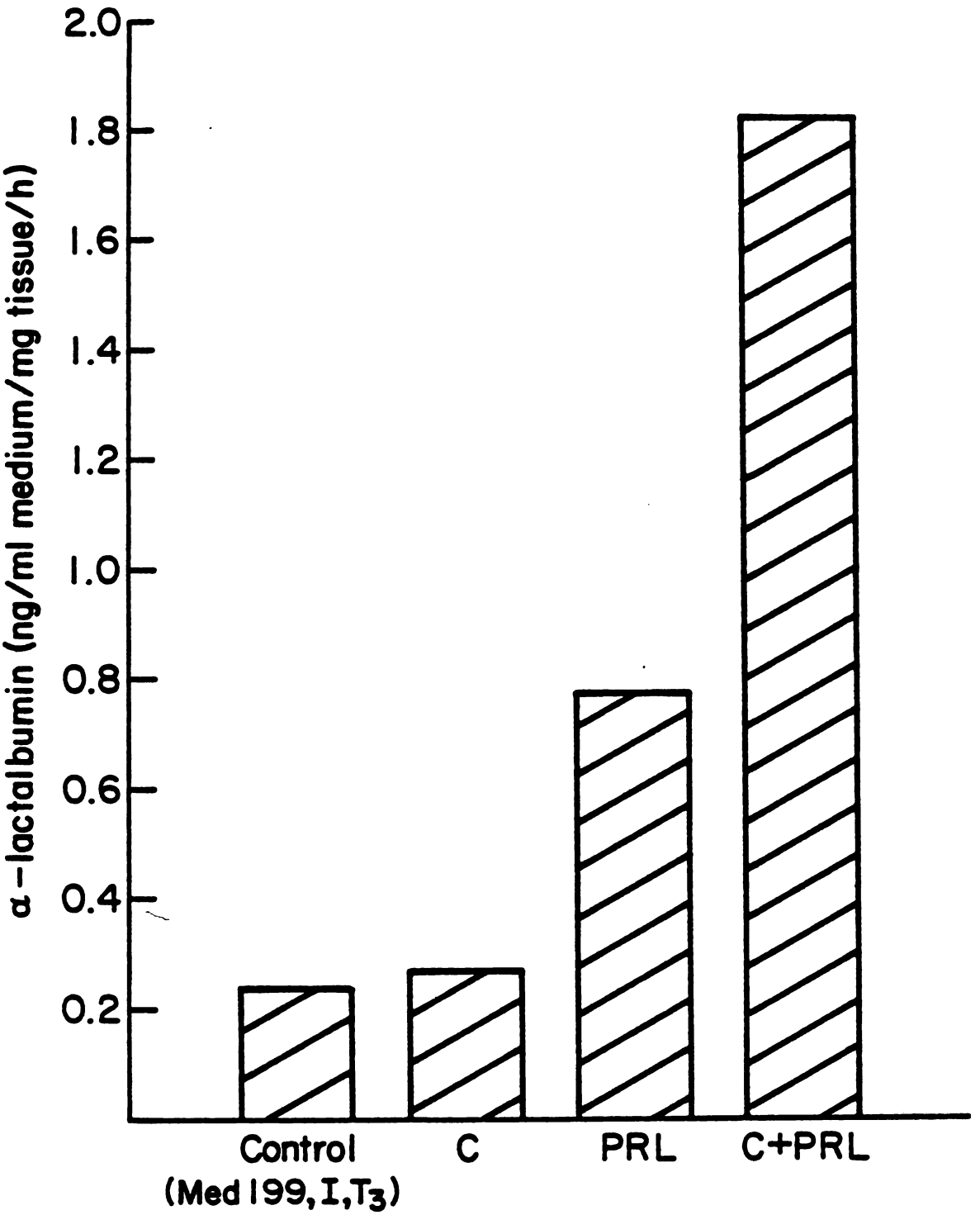
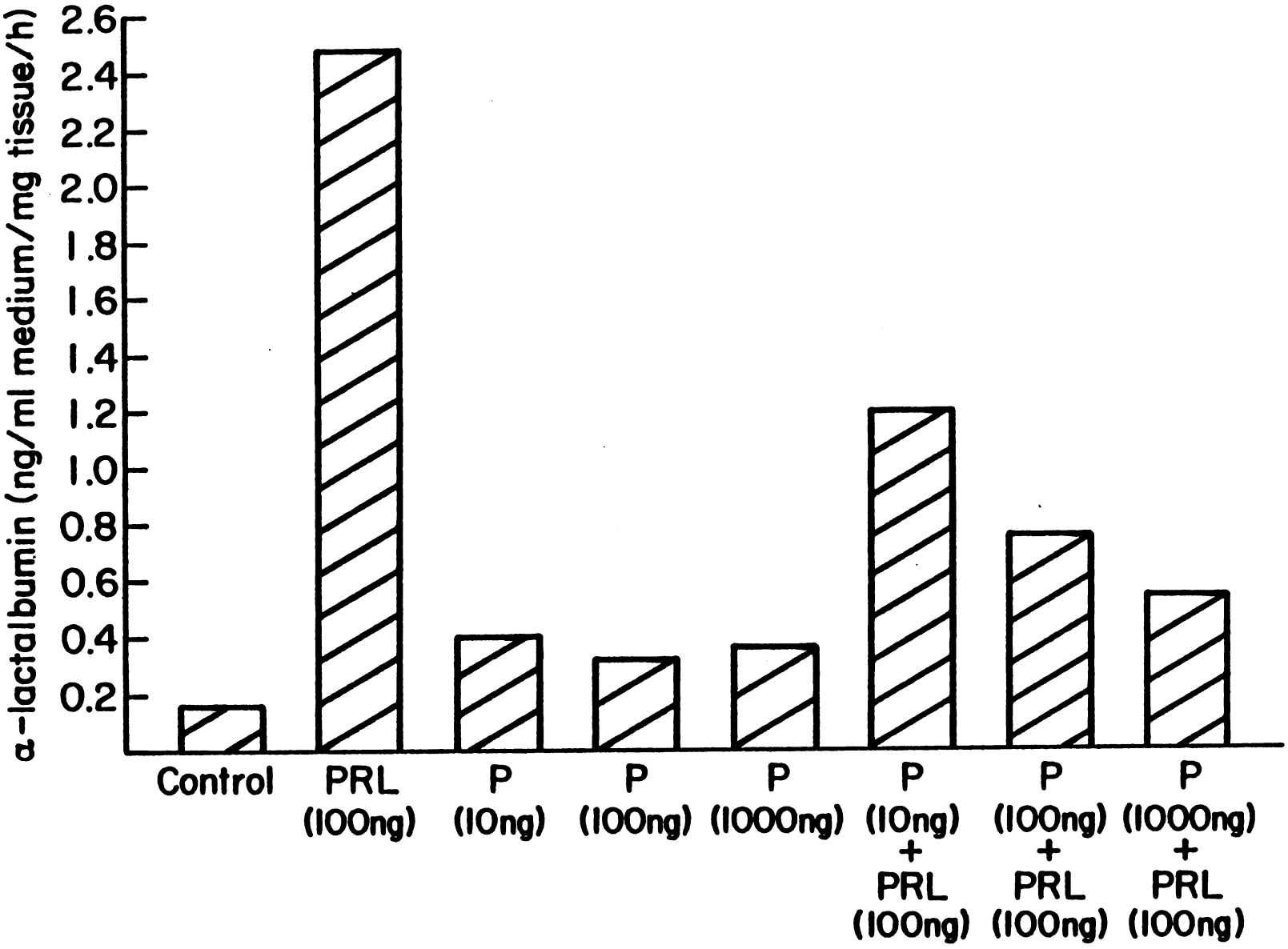

α-lactalbumin (ng/ml medium/mg tissue/h)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Control
(Med 199, I, T3)
C
PRL
C+PRL

Figure 7.--Mean concentrations of α-lactalbumin in medium from bovine mammary tissue cultured *in vitro* after exposure to 0, 10, 100 or 1000 ng progesterone/ml medium in the absence or presence of 100 ng prolactin/ml medium for 48 h. Standard error of difference between any two treatment means is .06.


α-lactalbumin (ng/ml medium/mg tissue/h)
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Control
PRL (100ng)
P (10ng)
P (100ng)
P (1000ng)
P (10ng) + PRL (100ng)
P (100ng) + PRL (100ng)
P (1000ng) + PRL (100ng)

($P<.005$) prolactin-induced secretion of α-lactalbumin from 2.44 to 1.20, .75 and .54 ng/ml medium/mg tissue/h, respectively.

Effect of Estradiol-17β on α-Lactalbumin Secretion

Figure 8 illustrates secretion of α-lactalbumin from mammary explants exposed to various concentrations of estradiol-17β in the presence or absence of 100 ng prolactin/ml medium. All doses of estradiol-17β increased rate of secretion of α-lactalbumin into medium approximately 3 fold, from .15 to approximately .46 ng α-lactalbumin/ml medium/mg tissue/h ($P<.01$). However, increasing the concentrations of estradiol-17β did not stimulate the mammary tissue to secrete increasing quantities of α-lactalbumin. Addition of 10, 100 or 1000 ng estradiol-17β progressively increased ($P<.001$) the prolactin-induced secretion of α-lactalbumin from 1.43 to 1.50, 1.88 and 2.33 ng α-lactalbumin/ml medium mg tissue/h, respectively.

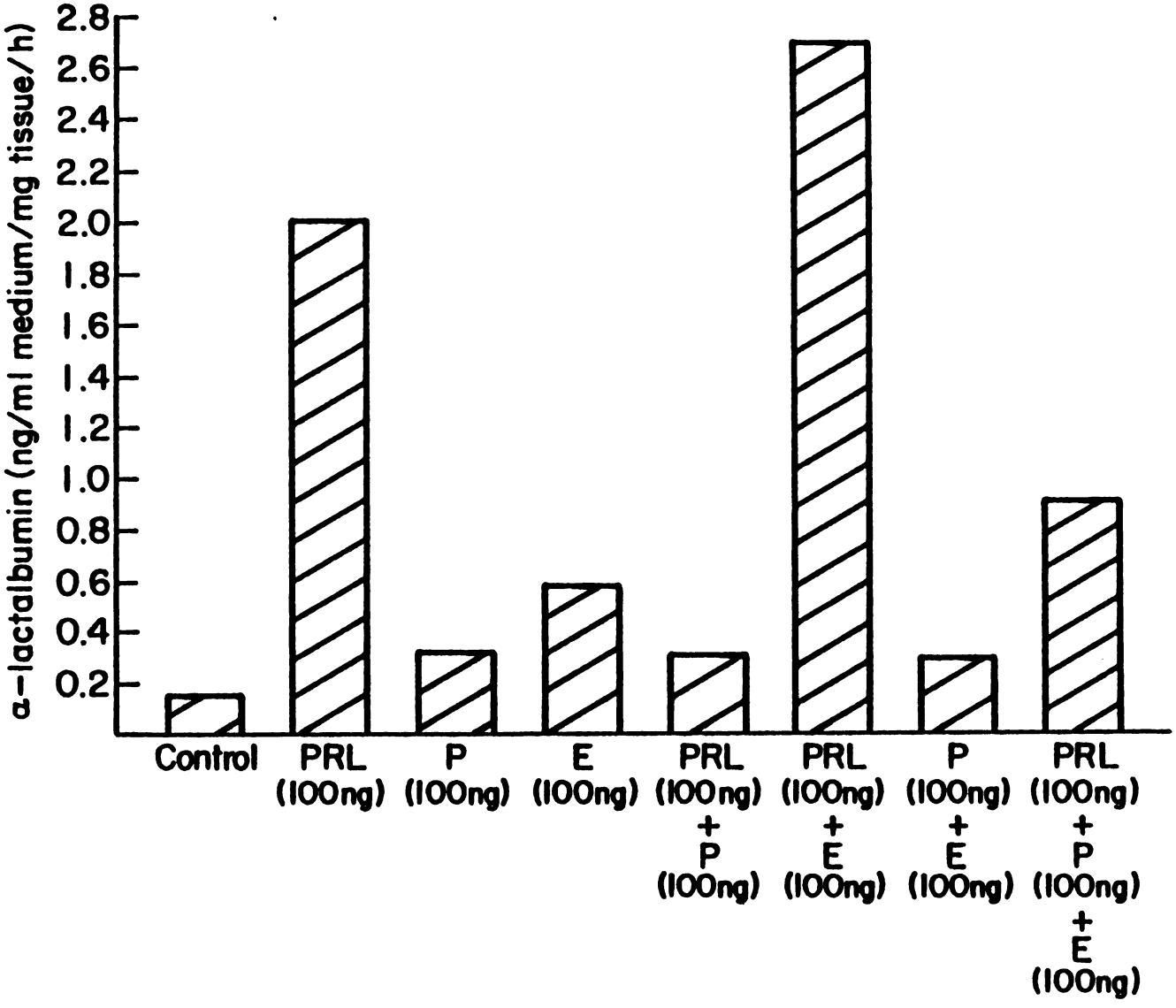
Effect of Estradiol-17β and Progesterone on α-Lactalbumin Secretion

Average concentrations of α-lactalbumin in medium secreted from mammary tissue exposed to either estradiol-17β, progesterone, or estradiol-17β plus progesterone are illustrated in Figure 9. Progesterone reduced prolactin-induced secretion of α-lactalbumin from 1.99 to .30 ng/ml medium/mg tissue/h ($P<.005$). Estradiol-17β enhanced the

Figure 8.--Mean concentrations of α-lactalbumin in medium secreted from bovine mammary tissue cultured *in vitro* after exposure to 0, 10, 100 or 1000 ng estradiol-17β/ml medium in the presence or absence of 100 ng prolactin/ml medium for 48 h. Standard error of difference between treatment means is .07.

α-lactalbumin (ng/ml medium/mg tissue/h)
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Control
PRL (100ng)
E (10ng)
E (100ng)
E (1000ng)
E (10ng) + PRL (100ng)
E (100ng) + PRL (100ng)
E (1000ng) + PRL (100ng)

Figure 9.--Mean concentrations of α-lactalbumin in medium secreted from bovine mammary tissue cultured *in vitro* in the presence or absence of 100 ng progesterone/ml medium, 100 ng estradiol/ml medium and/or 100 ng prolactin/ml medium for 48 h. Standard error of differences between treatment means is .06.


α-lactalbumin (ng/ml medium/mg tissue/h)
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Control
PRL (100ng)
P (100ng)
E (100ng)
PRL (100ng) + P (100ng)
PRL (100ng) + E (100ng)
P (100ng) + E (100ng)
PRL (100ng) + P (100ng) + E (100ng)

prolactin-induced secretion of α-lactalbumin from 1.99 to 2.69 ng/ml medium/mg tissue/h ($P<.01$). Addition of progesterone to mammary tissue cultures inhibited secretion of α-lactalbumin from mammary explants exposed to either estradiol-17β or estradiol-17β plus prolactin. Addition of progesterone decreased secretion of α-lactalbumin from 2.69 ng/ml medium/mg tissue in tissues exposed to estradiol-17β plus prolactin to .92 ng/ml medium/mg tissue/h ($P<.001$).

Effect of Growth Hormone on α-Lactalbumin Secretion

Figure 10 illustrates secretion rates of α-lactalbumin when explants were exposed to various concentrations of growth hormone in the presence or absence of prolactin. Growth hormone at 10 or 100 ng/ml medium did not significantly increase α-lactalbumin secretion over control levels ($P>.05$). At 1000 ng/ml, however, growth hormone increased secretion of α-lactalbumin from .87 to 1.60 ng/ml medium/mg tissue/h. Similarly, addition of 10 ng growth hormone to medium containing prolactin did not significantly increase α-lactalbumin secretion over prolactin-induced secretion alone ($P>.05$). However, 100 or 1000 ng growth hormone in the presence of prolactin significantly stimulated secretion of α-lactalbumin over that observed with prolactin alone, i.e. 3.14 vs. 3.77 ($P<.05$) and 4.23 ($P<.001$) ng α-lactalbumin/ml medium/mg tissue/h, respectively.

Figure 10.--Mean concentrations of α-lactalbumin in medium secreted from bovine mammary tissue cultured *in vitro* after exposure to 0, 10, 100 or 1000 ng growth hormone/ml medium in the absence or presence of 100 ng prolactin/ml medium for 48 h. Standard error of differences between treatment means is .03.

α-lactalbumin (ng/ml medium/mg tissue/h)
4.4
4.0
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
Control
PRL (100ng)
GH (10ng)
GH (100ng)
GH (1000ng)
GH (10ng) + PRL (100ng)
GH (100ng) + PRL (100ng)
GH (1000ng) + PRL (100ng)

DISCUSSION

It is generally believed that the periparturient surge of prolactin is essential for the secretion of copious quantities of milk in most mammals. Johke and Hodate (1978) administered ergocryptine and antiserum to bovine prolactin to cows two weeks before expected parturition. They found a 93% decrease in milk production immediately after parturition as compared to the previous lactation. Furthermore, previous milk production levels were not regained even 50 days after parturition. Similarly, Akers (1980) administered ergocryptine to one group of cows approximately 10 days prepartum to suppress prolactin secretion. He found that suppression of the parturition-induced surge of prolactin decreased subsequent daily milk yield by approximately 40% as compared with yield in control cows. He attributed reduction in milk production to reduced prolactin in circulation which in turn decreased mammary cell activity, especially concentrations of α-lactalbumin. Indeed, data from the present in vitro studies confirm Akers hypothesis that prolactin is one of the primary modulators of α-lactalbumin synthesis. Moreover, of all the hormones considered in the 'lactogenic complex' (estradiol-17β, cortisol and prolactin), prolactin

is probably the most important hormone for lactogenesis. This conclusion was based on the fact that estradiol-17β and cortisol synergized with prolactin to stimulate secretion of copious quantities of α-lactalbumin in mammary explant cultures, however, cortisol and estradiol-17β alone had relatively little effect on α-lactalbumin secretion.

Although prolactin has been shown to induce lactose synthetase activity in mammary tissue of various mammalian species including rat (Kuhn, 1968), mouse (McKenzie et al., 1971), and rabbit (Delouis and Denamur, 1972; Forsyth et al., 1972), the concentration of prolactin usually used to elicit a lactogenic response from mammary tissue ranged from 500 ng to 200 μg/ml medium (Forsyth, 1971). In the present studies as little as 25 ng prolactin per ml medium significantly increased α-lactalbumin secretion over control explants. Moreover, increasing quantities of prolactin in the medium progressively increased α-lactalbumin secretion from the mammary explants.

The high sensitivity of the bovine explants to relatively small concentrations of prolactin may be attributed to presence of triiodothyronine in the medium. Vonderhaar (1975) has shown that triiodothyronine enhances the ability of prolactin to stimulate α-lactalbumin activity as much as 5-fold in mouse mammary tissue cultured in vitro. Thus triiodothyronine probably should be considered limiting for mammogenesis and lactogenesis

in vitro; as yet, however, there is no evidence that thyroid hormones by themselves are lactogenic or limiting to the onset of lactation *in vivo* (Nelson and Tobin, 1937; Johke and Hodate, 1978).

Prolactin stimulates secretion of α-lactalbumin into medium from rat (Warburton *et al.*, 1979), mouse (Emerman *et al.*, 1977), human (Kleinberg, 1975) and other primate mammary tissues cultured *in vitro* (Kleinberg, Todd and Niemann, 1978). However, my study is the first to indicate that prolactin specifically stimulates *de novo* synthesis and secretion of α-lactalbumin from bovine mammary tissue into medium. The observation that concentrations of α-lactalbumin in tissue homogenates did not change throughout the duration of culture, yet copious quantities of α-lactalbumin were continuously released into media from explants exposed to prolactin, suggests that prolactin stimulates synthesis and secretion of α-lactalbumin. Moreover, tissue exposed to ^{3}H-leucine and prolactin for 3 h incorporated more than 10 times the quantity of ^{3}H-leucine as compared with tissues cultured in the absence of prolactin. This further indicates that prolactin specifically stimulates *de novo* synthesis of α-lactalbumin and reinforces the concept of Akers (1980) that prolactin may be the limiting hormone for subsequent milk production or lactogenesis in the cow.

Presence of progesterone prevents the onset of lactation in mice (Turkington and Hill, 1969), sheep (Hartmann et al., 1973), rabbits (Denamur and Delouis, 1972) and rats (Deis, 1968). I observed that the mere removal of mammary tissue from pregnant, nonlactating cows caused the release of copious quantities of α-lactalbumin from the excised tissue. One explanation for this phenomenon could be that the traumatization of the tissue associated with excision and manipulation of the explants when initially placed in culture caused the release of massive quantities of α-lactalbumin from the mammary tissue. Another explanation could be that the mammary tissue is removed from an environment in the pregnant cow in which there is an elevated concentration of progesterone (Smith et al., 1973) and immediately after biopsy the mammary tissue was placed in culture media without progesterone. Thus, the mammary tissue was removed from the tonic inhibitory effects of progesterone (Kuhn, 1969) which would be expected to initiate lactation.

Kuhn (1977) suggested that lactogenesis occurs when the mammary gland is released from inhibition by progesterone, and pushed by prolactational hormones, such as prolactin and cortisol. Indeed, bovine mammary tissue placed into medium containing prolactin plus progesterone inhibited the lactogenic effects of prolactin. However, increasing quantities of prolactin relative to progesterone

can override the tonic inhibitory effects of progesterone as seen also in sheep (Hartmann et al., 1973), mouse (Turkington and Hill, 1969), rat (Kuhn, 1969; Deis, 1968; Yokoyama et al., 1969) and rabbit (Delouis et al., 1974; Assairi et al., 1974) mammary tissue. Therefore, decreasing concentrations of progesterone in plasma at parturition is one of the major events leading to synthesis and secretion of copious quantities of milk in cattle and must occur before prolactin can exert its maximal lactogenic effects.

The mechanism by which progesterone inhibited α-lactalbumin secretion from mammary explants cultured in the presence of prolactin could be via inhibiting prolactin binding to mammary epithelia, as Djiane and Durand (1977) have shown in rabbit mammary tissue.

Increased mammogenesis begins shortly after conception and continues until early lactation in many species (Tucker, 1981). Feldman (1961) determined that lobule-alveolar differentiation of the mammary gland begins approximately 150 days after conception in the cow and is nearly complete by parturition. As parturition approaches, mammary secretory cells gradually acquire specific enzymes and cellular organelles necessary to synthesize and secrete copious quantities of milk. Therefore, hormones governing lobule-alveolar differentiation during mammogenesis are, in part, responsible for the secretion of copious quantities of milk during

lactogenesis. Thus, mammogenesis is tightly linked to lactogenesis. Results from the present study demonstrate that presence of either estrogen or progesterone alone stimulates α-lactalbumin secretion from mammary tissue. Perhaps the lactogenic response of the mammary explants to these steroids is a result of increased mammary cell proliferation or differentiation. This is not an unreasonable hypothesis considering that bovine mammary tissue is not fully differentiated until after parturition (Saacke and Heald, 1974; Akers, 1980), and that in the present experiment, mammary tissue was removed from cows that were 4-6 weeks prepartum. Moreover, both progesterone and estrogen stimulate alveolar growth of mammary tissue in various laboratory animals (Lyons, 1958; Mishkensky *et al.*, 1967; Trentin and Turner, 1948; Wood *et al.*, 1975) and cows (Sud, Tucker and Meites, 1968). The present data also confirm the observation of Nickerson *et al.* (1978) that estrogen and progesterone in media caused slight secretion of milk in the alveoli and enlarged luminal areas of bovine mammary tissue cultured *in vitro*.

The role of estradiol-17β in lactogenesis in the cow has been previously thought to be via increasing numbers of secretory cells in the mammary gland. This belief is based upon the following: 1) Throughout the majority of pregnancy concentrations of estrogen are elevated in serum of cattle (Smith *et al.*, 1973);

2) Formation of lobule-alveolar tissue in the mammary gland coincides with increased concentrations of estradiol and progesterone (Turner and Schultz, 1931; Turner and Allen, 1933); 3) Cultures of sheep mammary tissue require prior exposure (priming) with estrogen for 2 days before alveolar growth can be induced with other lactogenic hormones such as prolactin and cortisol (Jeulin-Bailly et al., 1973); 4) Administration of rather large concentrations of estradiol-17β and progesterone into cattle induce mammary growth and lactation (Collier et al., 1976). However, the present results are the first to indicate that estradiol-17β directly stimulates bovine mammary tissue to synthesize milk components. Thus, increased secretion of estradiol-17β during the periparturient period is important for lactogenesis because it; 1) stimulates secretion of prolactin in rats (Niswender et al., 1969; Chen and Meites, 1970) and cows (Padmanabhan and Convey, 1979); 2) directly stimulates mammary tissue to secrete milk components, i.e. α-lactalbumin (possibly by further increasing mammary tissue growth); 3) enhances prolactin's ability to stimulate synthesis of milk components; and 4) inhibits progesterone secretion from the corpus luteum, thereby decreasing progesterone concentrations in serum.

The mechanism by which estradiol-17β enhanced the ability of prolactin to stimulate α-lactalbumin may be via induction of prolactin receptor sites, as in the

rabbit (Sheth et al., 1978). However, Delouis (1975) was unable to show any lactogenic effect of estradiol-17β in the presence or absence of prolactin in rabbit or ewe mammary explants. But recently Bolander and Topper (1980) observed that in order for estradiol-17β to augment lactose synthetase activity in mouse mammary explants both triiodothyronine and prolactin must be present. The lack of triiodothyronine in Delouis' medium may explain why he was unable to show potentiation of prolactin's lactogenic effects by estradiol-17β. In a recent study, Delouis et al. (1980) observed that administration of estradiol benzoate at day 144 of pregnancy in sheep increased subsequent milk yields. Delouis suggested that the mechanism by which estradiol-17β increased milk yields may have been via enhancement of prolactin release from the pituitary, water imbibition of the mammary secretory cells (Ui and Mueller, 1963) and/or increased numbers of oxytocin receptors involved in milk ejection (Ollivier-Bousquet, 1976). Data from the present study, also suggests that estradiol-17β directly affects mammary tissue to enhance prolactin's actions. Therefore, estradiol-17β is necessary for the initiation of secretion of copious quantities of milk in cattle.

It is generally believed that cortisol is essential for the first stage of lactogenesis. Cortisol stimulates formation of organelles within the mammary cell and is

necessary for secretion of voluminous quantities of milk (Mills and Topper, 1970). Data from the present studies indicate that cortisol enhances the lactogenic effects of prolactin but has no lactogenic effects when added alone into medium supplementing the bovine mammary tissue. These results are similar to findings of Jeulin-Bailly *et al.* (1973) that alveolar lumina were enlarged but contained no secretion when ovine mammary explants were supplemented with insulin and cortisol. The present results also agree with the hypothesis that cortisol potentiates the action of prolactin on milk component synthesis in rabbit (Devinoy *et al.*, 1978) and rat (Rosen *et al.*, 1975) mammary tissue, but has little lactogenic effect when exposed to mammary tissue by itself. Since cortisol alone did not significantly increase α-lactalbumin secretion from bovine mammary explants in the present study, it is possible that the mechanism by which cortisol acted to stimulate lactogenesis in heifers (Tucker and Meites, 1965) may have been via potentiating prolactin's lactogenic actions directly upon mammary tissue.

Addition of growth hormone into medium in the present studies in quantities normally found *in vivo* (10 or 100 ng/ml) did not increase α-lactalbumin secretion over controls. This corresponds with the hypothesis that growth hormone is not lactogenic *per se*, but synergizes

with other lactogenic hormones to initiate lactation (Tucker, 1979). These data partially agree with Jeulin-Bailly *et al*. (1973) that addition of growth hormone to media containing insulin, steroids and prolactin elicited a greater secretory response in heifer mammary tissue cultured *in vitro* than tissue cultured in this same medium without prolactin. Although any additional lactogenic effects that growth hormone has over prolactin when mammary tissue was cultured in the presence of both prolactin and growth hormone may be due, in part, to prolactin contamination of the growth hormone preparations. In many previous studies in which growth hormone was used to initiate lactogenesis, the concentrations of growth hormone were sometimes in excess of 200 µg/ml (Rivera, 1964; Nandi, 1961; Barnawell, 1965). If 200 µg of a growth hormone preparation contains as little as one-half of a percent 'prolactin' contaminant, approximately 1000 ng would remain in the growth hormone preparation.

Thus, the importance of the periparturient surge of growth hormone on lactogenesis remains to be elucidated. It is possible that the periparturient surge induces growth hormone receptor sites which are necessary for the galactopoitic effects of growth hormone in the subsequent lactation. Although growth hormone did not show any lactogenic effects in the present *in vitro* study, this does not rule out the possibility that growth hormone is

lactogenic *in vivo*, since growth hormone may act on the mammary gland *in vivo* through a mediator such as the somatomedins.

Twenty years have past since Meites (1961) stated that the outstanding problem that remains in the artificial induction of lactation in dairy animals is the great variation in lactational response. This remains true today. The reason(s) for the wide variation in milk production from cows artificially induced into lactation has not been extensively investigated. Studies measuring serum estrogens and progesterone concentrations during the treatment period failed to demonstrate any relationship between hormone concentrations and subsequent milk production (Monk *et al.*, 1973). Since progesterone can tonically inhibit prolactin secretion from the pituitary (Karg and Schams, 1974) and inhibit prolactin's ability to induce prolactin receptors (Djiane and Durand, 1977) on mammary tissue it is probable that the great variability in milk production is due to variability of prolactin secretion, metabolic clearance rate or its action directly upon the mammary tissue. Indeed, Collier *et al.* (1977) injected nonpregnant, nonlactating cattle with estradiol-17β and progesterone followed by reserpine (to elevate plasma prolactin concentrations) or saline. Their results indicated that animals given reserpine had higher peak milk yields and greater milk production than cows given

estradiol-17β, progesterone and saline. Thus, prolactin may be limiting to subsequent milk production in artificially-induced as well as natural-onset lactations.

An obvious advantage of *in vitro* tissue cultures is that isolation of the tissue from the body allows one to test the individual direct effects of various hormones on specific biochemical or morphological responses. However, the amplitude of the response exhibited by the mammary tissue is dependent upon various factors including the degree of maturation of the tissue, the quantity of hormone employed in the medium, the biochemical or morphological response that was selected to be measured, and the amount of time after initiation of culture. For example, Chatterton *et al.* (1975) conducted ultrastructural examinations of mammary tissue from rats during the 24 h period prior to parturition and demonstrated that proteins and lipids accumulated within the secretory cells until approximately 8 to 12 h prior to parturition at which time these milk components were secreted into the alveolar lumen. They suggested that the process associated with milk secretion from the epithelial cells is initiated independent of intracellular milk synthesis. Thus, it is possible that the hormones controlling intracellular synthesis of milk are different from those that control milk secretion into the alveolar lumen prior to parturition. In a recent study, Ono and Oka (1980) showed that

differences in hormonal mileau may account for differences in milk protein synthesis vs. secretion. They cultured mouse mammary tissue in medium containing prolactin, insulin and 10^{-6}M cortisol for 2 days, and found α-lactalbumin in medium in approximately the same concentrations as found in mammary tissue homogenates. In the presence of $3x10^{-8}$M cortisol over 70% of total α-lactalbumin was retained in tissue. Thus, cortisol exerted different actions on the accumulation of α-lactalbumin in mammary tissue depending upon dose employed. The concentration of cortisol employed in the present experiments allowed secretion of copious quantities of α-lactalbumin into the media and accumulation of α-lactalbumin in tissue was not evident throughout the duration of culture. However, α-lactalbumin synthesis inevitably declined 48 h after initiation of culture which agrees with data from other groups of researchers utilizing a similar culture system (Anderson and Larson, 1970; Kinsella, 1968; Collier et al., 1977). This decline in α-lactalbumin synthesis is not likely to be due to end-product feedback inhibition since lactose does not suppress synthesis of its precursor enzymes, i.e., galactosyl transferase or α-lactalbumin, in rat (Palmiter, 1969) or mouse (Kuhn, 1969) mammary tissue.

Appearance of various proteins, sugars and fatty acids that are specific for milk production have been found

in mammary tissue as early as 2 to 4 weeks prior to parturition (Mellenberger et al., 1973; Hartmann, 1973; Collier et al., 1976). However, Akers (1980) demonstrated that many of the enzymes necessary for milk secretion were either not detectable or had relatively low activities 10 days prepartum. However, activity of these enzymes increased several times by 10 days postpartum. Results from my studies indicate that α-lactalbumin is found in and released from mammary tissue obtained from cows that were at least 6 to 8 weeks prepartum. Although the physiological importance of α-lactalbumin during this stage of pregnancy in mammary tissue is not known, it is apparent that mammary tissue even at this stage of pregnancy contains enzymes necessary to secrete copious quantities of milk. Thus, presence of α-lactalbumin, as measured by radioimmunoassay, occurs in cow mammary tissue much earlier than others have reported. One reason that we found α-lactalbumin in mammary tissue at this stage of pregnancy and others have not, could be because radioimmunoassay of α-lactalbumin is approximately 10 to 100 times more sensitive than enzymatic assays (Kleinberg et al., 1978; Brew et al., 1978), and that researchers utilizing enzymatic assays could not detect lactose synthetase components because the assays utilized were not sufficiently sensitive to detect α-lactalbumin at this stage of pregnancy.

To briefly summarize the discussion, during early stages of pregnancy estrogen and progesterone stimulate mammogenesis in the cow. Progesterone tonically inhibits lactogenesis by inhibiting prolactin secretion from the pituitary as well as inhibiting prolactins' actions at the mammary secretory cell via inhibiting induction of prolactin receptor sites. As parturition approaches milk synthesis occurs in spite of the high concentrations of progesterone in serum. This could be because the mammary secretory cell is developing organelles for the synthesis of milk in response to cortisol, and also because increasing quantities of estradiol-17β in serum may potentiate prolactin's lactogenic effects on the mammary cell. Prior to parturition, the decline in serum progesterone allows the mammary secretory cell to respond to the 'lactogenic complex' of hormones which include estradiol-17β, prolactin and cortisol, thus stimulating the mammary gland to secrete copious quantities of milk.

Based upon the results of the present study, I conclude that prolactin, cortisol and estradiol-17β are essential for secretion of copious quantities of milk from bovine mammary tissue, provided progesterone is absent.

SUMMARY AND CONCLUSIONS

The role of prolactin, cortisol, estradiol-17β, growth hormone and progesterone in synthesis and secretion of α-lactalbumin were studied utilizing explants of bovine mammary tissue cultured *in vitro*. Alpha-lactalbumin was used as a biochemical indicator of lactogenesis because; 1) hormones regulate α-lactalbumin synthesis, 2) intracellular synthesis and secretion of α-lactalbumin into the alveolar lumen can be quantified by a sensitive radioimmunoassay, and 3) accumulation of lactose does not inhibit α-lactalbumin synthesis by end-product feedback inhibition.

Mammary tissue, obtained from pregnant, multiparous, nonlactating cows ranging from 1 to 8 weeks prepartum, was diced into explants, then exposed to medium 199 containing .65 ng triiodothyronine, 5 μg insulin/ml medium and various combinations and doses of prolactin, cortisol, progesterone, estradiol-17β and growth hormone for 72 h. Alpha-lactalbumin was quantified in tissue and media by radioimmunoassay or sodium dodecyl sulfate (SDS) gel electrophoresis.

Mammary tissue obtained from all cows contained considerable quantities of α-lactalbumin, but several serial washes of the explants prior to incubation reduced

expulsion of α-lactalbumin into medium. Since α-lactalbumin is the rate-limiting enzyme for lactose synthesis, and lactose synthesis is highly correlated with milk yield, I concluded that mammary tissue contains key milk synthesizing components as early as 2 months prior to parturition.

Mammary explants exposed to 100 ng prolactin/ml medium for 72 h secreted approximately 3.2 and 3.8 times the quantity of α-lactalbumin into medium at 48 and 72 h after initiation of culture, respectively, than explants cultured in the absence of prolactin. Greatest secretion of α-lactalbumin in response to prolactin occurred between 24 and 48 h after initiation of culture. However, concentrations of α-lactalbumin in mammary explants exposed to prolactin were not significantly different ($P>.05$) from concentrations of α-lactalbumin found in control explants. Furthermore, quantities of α-lactalbumin found in homogenates of tissues exposed to either 0 or 100 ng prolactin/ml medium did not significantly change throughout the 72 h duration of culture. These results, coupled with results from time-course studies in which ^{3}H-leucine was incorporated into α-lactalbumin indicated that; 1) intracellular synthesis and secretion of α-lactalbumin into alveolar lumena occurred throughout the duration of culture, and although synthesis of α-lactalbumin declined after 48 h of culture, there was no indication that

secretion was impaired, and 2) relatively small quantities of prolactin (25 ng/ml) stimulate synthesis and secretion of α-lactalbumin from bovine mammary tissue cultured in vitro.

In the absence of prolactin, .5 μg cortisol in media had no effect on α-lactalbumin secretion from mammary tissue ($P>.05$) over controls. However, cortisol synergized with prolactin to stimulate secretion of copious quantities of α-lactalbumin. These data provide conclusive evidence that cortisol does not directly stimulate the mammary gland to induce the lactogenesis but cortisol does enhance the ability of prolactin to stimulate secretion of copious quantities of milk from bovine mammary tissue.

Addition of various concentrations of either estradiol-17β or progesterone into medium stimulated secretion of greater concentrations of α-lactalbumin into media than control explants ($P<.05$). However, addition of both estradiol-17β and progesterone into media decreased α-lactalbumin secretion from bovine mammary explants as compared with explants receiving estradiol-17β alone ($P<.01$). I attributed the lactogenic effect of estrogen and progesterone to increased numbers of mammary cells. The combination of estrogen plus progesterone caused secretion of less α-lactalbumin from bovine mammary tissue than quantities measured when either of these steroids were used alone.

Addition of increasing concentrations of progesterone into media containing prolactin progressively decreased the ability of prolactin to secrete α-lactalbumin from bovine mammary tissue into the media ($P<.001$). These results suggest that progesterone tonically inhibits α-lactalbumin secretion during lactogenesis. Increasing concentrations of estradiol-17β progressively increased the ability of prolactin to secrete α-lactalbumin from bovine mammary tissue. Consequently, I conclude that the periparturient surge of estradiol-17β potentiates the effects of prolactin on the mammary gland. Addition of progesterone into media containing estradiol-17β and prolactin dramatically reduced prolactin's ability to secrete α-lactalbumin from mammary tissue. Collectively, these data indicate that prolactin is the rate limiting hormone of lactogenesis in the cow, and cortisol and estradiol-17β are necessary for secretion of maximal quantities of milk. These results further support the concept that progesterone tonically inhibits onset of lactation, however, increasing concentrations of the lactogenic complex, i.e., estradiol-17β, cortisol and prolactin, override this progesterone block.

Growth hormone in concentrations normally found in periparturient females (10 ng/ml serum) did not stimulate α-lactalbumin from mammary tissue over control levels ($P>.05$), nor did 100 ng/ml of growth hormone affect

α-lactalbumin secretion. Addition of 100 or 1000 ng growth hormone to medium containing prolactin augmented prolactin-induced α-lactalbumin secretion. I concluded that the elevation in growth hormone in serum prior to parturition is probably not great enough to directly stimulate the mammary cell to secrete copious quantities of milk at parturition.

APPENDICES

APPENDIX A

BUFFER SOLUTIONS

Tris-Salt Buffer (pH 7.3)

Trizma Base	25 mM
NaCl	25 mM
$MgCl_2$	5 mM
Triton X-100	0.5%

Tris-Sucrose Buffer (pH 7.3)

Sucrose	.25 M
Trizma HCl	.227 M
Trizma Base	7 mM
Glutathione	1 mM
Disodium EDTA	1 mM

APPENDIX B

STACKING SODIUM DODECYL SULFATE (SDS) GEL ELECTROPHORESIS

Advantages:

1. This method gives extremely high resolution.
2. Complete cellular extracts may be applied.
3. Samples of up to 1 ml may be applied.
4. Samples with high salt concentrations may be used.
5. Allows molecular weight of constituents to be estimated.

Reagents:

1. Electrode Buffer: 9.1 g Tris base, 43.2 g glycine, 3.0 g SDS (BioRad). Adjust pH to 8.3 and volume to 3 liters.
2. Sample Buffer (5x): 16 ml glycerol, 1.5 g Tris base, 4 mg Bromo Phenol blue. Adjust pH to 6.8 and volume to 31 ml. Add 2.2 ml 2-mercaptoethanol to 7.8 ml sample buffer.
3. Separation Gel Buffer (1.5 M Tris HCl, 0.4% SDS): 18.17 g Tris HCl, 4 ml 10% SDS. Adjust pH to 8.8 and volume to 100 ml with distilled water. Filter through Millex filter.

4. Spacer Gel Buffer (0.5 M Tris-HCl, 0.4% SDS): 6.06 g Tris HCl, 4 ml 10% SDS. Adjust pH to 6.8 and volume to 100 ml with distilled water.
5. 2% Ammonium persulfate solution: 100 mg ammonium persulfate in 5 ml distilled water.
6. 30% Acrylamide, 0.8% Methylene Bisacrylamide solution: 30 g acrylamide (BioRad), 0.8 g methylene bisacrylamide (Merck) dissolved in and adjusted to 100 ml with distilled water. Filter through Whatman No. 1.
7. Reservoir Buffer: 200 ml of Tris glycine buffer (4x), 20 ml of 10% SDS, and distilled water to 2,000 ml.
8. Tris-glycine buffer (4x): 12 g Tris-base and 57.6 g glycine. Make up to 1,000 ml with distilled water.
9. TEMED-neat.
10. Gel Tubes: 15 cm long, 0.5 cm I.D. Soak overnight in Chromerge solution. Rinse well. Dry. Silanize, bake at 250 C, 1-2 h. Mark with pen 10 and 11 cm from gel bottom.

Preparation of Gels:

A. 1. Separation Gel (12.5%):

a. 10 ml separation gel buffer
b. 8.3 ml 30% acrylamide 38:1
c. 1.7 ml water
d. 0.3 ml 2% ammonium persulfate solution
e. 5 µl TEMED

2. Degas a, b and c for 15 min.

3. Add d and e to degassed mixture.

4. Parafilm lower end of gel tube.

5. Pour separation gel to 10 cm mark of gel tube.

6. Polymerize gel one h with isopropanol on top of the gel, this keeps the top of the gel flat as it polymerizes.

B. 1. Spacer Gel (3%):

a. 1.94 ml distilled water
b. 2.5 ml spacer gel buffer
c. 0.5 ml 30% Acrylamide 38:1
d. 65 µl 2% ammonium persulfate
e. 5 µl TEMED

2. Degas a, b and c for 15 min.

3. Add d and e to degassed mixture.

4. Rinse isopropanol off from polymerized separation gel with water 4 or 5 times, then with reservoir buffer 4 or 5 times.

5. Add 1 cm spacer gel and allow to polymerize for one h.

C. 1. After removing parafilm, place gels in the electrophoresis unit and make sure there are no air bubbles trapped on the bottom or top of gels.

2. Plug unused spaces for tube gels and fill upper unit with reservoir buffer.

3. Apply samples to tube gels and perform electrophoresis at 2 m Amp per tube for 1 h, then at 4 m Amp per tube for 3 to 4 h.

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