CORTICOID RESPONSE TO PREGNANCY, PARTURITION, MILKING AND NONSPECIFIC STIMULI

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ABSTRACT

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By

Valdin G. Smith

Plasma corticosterone concentration in rats killed on day 11 of pregnancy averaged 28.3 µg%, significantly less ($p < 0.05$) than comparable averages for rats killed on day 19 of pregnancy (40.3 µg%) and at parturition (40.9 µg%). Plasma corticosterone concentration in rats sacrificed on day two or 15 of lactation was unchanged relative to the comparable value at parturition.

Serum corticoid concentration in cows averaged 5.0 ng/ml from 26 to one day prepartum then increased ($p < 0.005$) to 10.3 and 16.7 ng/ml, 12 hours before and at parturition respectively. By approximately 12 hours postpartum serum corticoid decreased ($p < 0.001$) to 5.1 ng/ml and averaged 5.6 ng/ml during the early postpartum period. Increased corticoid concentration near to or at parturition is suggestive of its involvement in the initiation of parturition and lactation.

Rats continually suckling two or six-pup litters were sacrificed on day eight of lactation. Plasma corticosterone concentration averaged 23.8 and 29.3 µg% for rats with two and six-pup litters respectively. Removal of litters for 12.5 hours from rats suckling two or six pups decreased plasma corticosterone concentration to 19.7 and 20.2 µg% respectively. However, only in the six-pup group was this significant ($p < 0.05$). Following 12.0 hours nonsuckling, 0.5 hour of suckling increased plasma corticosterone concentration 100.5 and 334.0% in rats suckling two and six pups respectively, relative to corresponding nonsuckled controls. Neither litter size nor acute suckling affected adrenal ascorbic acid concentration. Mammary weight of rats nursing six pups averaged 12.6 g, greater ($p < 0.01$) than comparable average of rats suckling two pups (7.6 g). When litters were removed for 12.5 hours, accumulation of milk resulted in heavier mammary weight but only in six-pup group was this significant ($p < 0.01$). Ovarian weight of rats suckling two-pup litters averaged 78.1 mg, significantly greater ($p < 0.05$) than comparable weight of rats with six-pup litters (70.3 mg). Presumably the stimuli generated by the greater suckling intensity suppressed ovarian weight.

Average serum corticoid concentration in milked cows was increased ($p < 0.05$) at five and 15 minutes after the start of milking relative to the premilked average. By

60 minutes postmilking serum corticoid had declined to premilked concentration. Analysis of the corticoid response of cows which were not milked but sampled while their pairmates were milked revealed a significant ($p < 0.001$) interaction between days and time of sampling. In nonmilked cows on day one, there was no change in serum corticoid concentration across the sampling period. However, when the milked cows of day one served as nonmilked controls on day two there was a significant increase ($p < 0.05$) in corticoid concentration, five minutes before milking had begun which persisted to 15 minutes postmilking. The results suggest that milking per se and exteroceptive stimuli are capable of eliciting corticoid release in the cow.

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INTRODUCTION

With techniques developed to measure hormones, dairy scientists are aware that not only proper breeding, feeding and management regulate milk production but hormones are also involved. One method to study hormones *in vivo* is to measure their concentrations and responses during different physiological states.

The purpose of these experiments was to evaluate adrenal response to pregnancy, parturition, milking and nonspecific stimuli, hoping that on completion, further relevant information could be contributed to achieve better efficiency in dairy production.

Adrenal glucocorticoids are involved in initiating parturition and lactation. Administration of glucocorticoid to lactating rats can prolong lactation suggesting that glucocorticoid is rate limiting during advanced lactation in rats. Future wide scale use of glucocorticoid in the dairy industry to induce parturition may occur since parturition could be induced at the most convenient time to the farm enterprise. In addition, understanding the mechanisms by which adrenal secretions are controlled may provide basic information required for manipulation

of corticoid levels to achieve increased milk production and growth.

REVIEW OF LITERATURE

A. Adrenal Embryology

1. General

In most animals the adrenal gland consists of a cortex and medulla, which are anatomically and functionally distinct. In lower vertebrates the cortex and medulla remain spatially separate throughout life, but as the phylogenetic scale is ascended there is intimate association of cortex and medulla (Arey, 1963). Embryologically, the cortex and medulla originate from different sources. Nelsen (1953) and Shumway and Adamstone (1954) observed that the adrenal cortex originates from mesodermal tissue in the region where the genital ridge subsequently appears. The medulla arises from ectodermal tissue in the neural crest as do cells forming the sympathetic ganglion. Following establishment of the cortex, migration of chromaffin cells establishes the medulla within the surrounding cortex. After the cortical primordium becomes established it is surrounded by more compactly arranged cells which originate from the same area as the first cortical analage (Arey, 1963). The second group of cells forms a permanent cortex while the original mass forms the provisional or fetal cortex.

2. Fetal Cortex

The fetal cortex is the first mass of cortical cells laid down and contributes the major portion of adrenal gland weight at birth. During the early postnatal period there is a concurrent regression of cells of the fetal cortex and growth of new cells establishing the permanent cortex. Benner (1940) described a narrow outer zone of new cortex, a wide middle zone of fetal cortex and few medullary cells in human adrenal glands at birth. By the third neonatal week about four-fifths of the cells of the fetal cortex had degenerated. In three week old rats Howard (1938) observed a juvenile cortex which was later transformed into the zona reticularis. Similarly, Nicander (1952) observed a fetal cortex in cattle which contributed the major portion of adrenal gland weight at birth and which was transformed into the zona reticularis shortly after birth. Howard (1938) established the relationship between the fate of the adrenal X-zone of rodents and gonadal steroids when he demonstrated disappearance of this zone at puberty in males and at first pregnancy in females and re-establishment of this zone in postpubertal castrate rats. Dickson (1970) observed an X-zone in adrenals of juvenile mice and rabbits which was replaced by the zona reticularis at puberty in males and during first pregnancy in females.

The importance of the fetal cortex to postnatal adrenal physiology is still to be elucidated.

B. Adrenal Anatomy and Morphology

1. Gross

Shape of the adrenal glands vary among species and also between right and left glands. Generally, in mammals the adrenal glands are ellipsoid organs encapsulated by a tough layer of connective tissue and are located on the dorsal wall of the abdomen anterior to the kidneys at the level of the first lumbar vertebrae. Blood supply to the adrenal consists of the superior, middle and inferior adrenal arteries which are branches of the inferior phrenic, aorta and renal artery, respectively. Venous drainage is by the left and right adreno-lumbar veins to the renal vein and vena cava, respectively. The nerve supply to the medulla consists of preganglionic fibers from the splanchic and suprarenal plexus of the autonomic system. The adrenal cortex is without nervous connection.

2. Microscopic Anatomy

On the basis of cell arrangement the adrenal cortex is divided into three zones: (1) a thin outer zone--zona glomerulosa; (2) a thick middle zone--zona fasciculata; and (3) an inner zone--zona reticularis. These zones are reported to be distinct in three-week-old calves (Weber et al., 1950) and rats (Jackson, 1919).

Nicander (1952) reported numerous lipid granules in the zona fasciculata and inner one-half of the zona glomerulosa. Lipid granules probably serve as precursor for steroid synthesis since they accumulate during periods of gland inactivity but are rapidly depleted following stimulation of steroid synthesis (Dickson, 1970).

The site of regeneration of adrenal cortical cells has been a source of controversy for years. Jackson (1919) observed greatest mitotic activity in the zona glomerulosa and outer most part of the zona fasciculata. However, Zwemer et al. (1938) reported that the adrenal cortex grows only from subcapsular cells; newly formed cells migrating inward and becoming degenerate in the zona reticularis. Jayne (1953) reported the presence of mitotic figures throughout the adrenal cortical region in rats up to three weeks old but not thereafter. However, Pauly (1956) described cell division as being limited to the zona glomerulosa and outer portion of the fasciculata, i.e. no mitosis in the capsule. Salmon and Zwemer (1941) presented evidence in support of the hypothesis that the cortex grows from capsular cells which migrate to the reticular area and become degenerated. Leeson and Leeson (1970) reported the presence of mitotic figures in all zones and degenerating cells in zones other than the zona reticularis, thereby making it doubtful that the zona reticularis is the only site of cell degeneration.

C. Adrenocortical Secretions

1. Hormone Classification

Steroid hormones produced by the adrenal cortex can be classified into three broad groups.

1. Mineralocorticoids--control electrolyte and water balance.
2. Glucocorticoids--part of carbohydrate metabolism regulatory mechanism.
3. Sex hormones--mediate and regulate processes of reproduction and lactation.

The dominant mineralocorticoid in most mammalian species is aldosterone while the dominant glucocorticoid is variable according to species. Singer and Stack-Dunne (1955) reported the primary glucocorticoid of rats to be corticosterone. Bush (1953) observed the presence of both cortisol and corticosterone in bovine blood with cortisol predominant.

2. Secretory Specialization of Cortical Zones

Evidence obtained by establishing deficiencies using hypophysectomy or sodium deprivation indicates a functional specialization of the adrenocortical zones. Deane *et al.* (1948) observed a reduction in adrenal gland weight and glucocorticoid secretion following hypophysectomy in rats. Histological analysis of these glands revealed atrophy of the zonae fasciculata and reticularis but retention of the

secretory appearance of the zona glomerulosa suggesting the zonae fasciculata and reticularis as sites of glucocorticoid secretion and the zona glomerulosa as the site of mineralocorticoid secretion. Eisenstein and Hartroft (1957) reported increased aldosterone and decreased corticosterone secretion in sodium-deficient rats. These results are not equivocal, however, since Deane et al. (1948) had previously reported reduced aldosterone secretion in rats made sodium-deficient.

Results obtained from in vitro incubation of bovine adrenal tissue suggested that aldosterone is produced by the zona glomerulosa, cortisol by the zona fasciculata and corticosterone by both the zonae fasciculata and glomerulosa (Ayres et al., 1956; and Stachenko and Giroud, 1959). Giroud et al. (1954) used in vitro techniques to establish the zona fasciculata as the only site of corticosterone secretion in the rat.

3. Control of Adrenocortical Secretion

Bergner and Deane (1948) reported atrophy of the zonae fasciculata and reticularis but not the zona glomerulosa in hypophysectomized rats. These results suggest that glucocorticoid but not mineralocorticoid secretion is under pituitary control. They also demonstrated an increase in 11-oxygenated steroids but not aldosterone after treatment of rats with adrenocorticotrophic hormone (ACTH). In contrast, Singer and Stack-Dunne (1955) and Sheppard et al.

(1963) reported increased corticosterone and aldosterone secretion after administration of exogenous ACTH to rats. Davis (1961) observed increased aldosterone secretion following surgical stress which could be inhibited by nephrectomy. This result would indicate a possible intermediary role for the kidney in controlling aldosterone secretion.

D. Biosynthesis of Adrenal Cortical Steroids

Cholesterol appears to be the primary precursor for synthesis of adrenal cortical steroids.

Before discussing synthesis of the primary adrenal cortical steroids, features which are common to all of them are worth mentioning. Their common features are:

1. All contain a perhydrocyclopentanophenanthrene nucleus.
2. All are composed of 21 carbon atoms.
3. A double bond is present between carbon atoms 4 and 5.
4. All bear an oxygen on C-3.

In the synthesis of corticoids from cholesterol the first C-21 product is pregnenolone (Stone and Hetcher, 1954; Hayano et al., 1956; Dorfman, 1957; and Grant, 1968). Pregnenolone was reported by the former investigators to be rate limiting to steroid biosynthesis presenting the possibility that conversion of cholesterol to pregnenolone

may be a control point. Oxidation of pregnenolone at C-3 by a cytoplasmic enzyme (3β-hydroxy-dehydrogenase) produces progesterone. Progesterone then undergoes a series of hydroxylations at positions 21, 17, and 11 to yield corticosterone and/or cortisol, and at C-18 to produce aldosterone. The 17α-hydroxylating system predominates in animals which produce cortisol as the dominant glucocorticoid. In rats 21-hydroxylation is dominant yielding corticosterone as the primary glucocorticoid (Grant, 1968).

Hydroxylating enzymes require molecular oxygen and reduced nicotinamide adenine dinucleotidephosphate (NADPH) as cofactors. Haynes and Berthet (1956) hypothesized how the adrenal acquires NADPH for steroid synthesis. According to their hypothesis, stimulation of the adrenal cortex with ACTH causes an increase in 3'5' adenosine monophosphate (AMP). This nucleotide then stimulates an active phosphorylase which catabolizes glycogen to glucose-1-phosphate which is rapidly converted to glucose-6-phosphate. Subsequent dehydrogenation of glucose-6-phosphate generate NADPH which stimulates corticosteroid synthesis. Later investigation of Haynes (1958, 1959), Haynes *et al.* (1960), Grahame-Smith *et al.* (1967) and Bell *et al.* (1970) lend credence to this hypothesis, as addition of ACTH to quartered adrenals *in vitro* caused an increase in cyclic AMP and corticosterone synthesis.

Specific localization of enzymes in the adrenal cortex was proposed by Hayano et al. (1956). They found that in the zonae fasciculata and reticularis, labeled progesterone was primarily converted to hydrocortisone while in the glomerulosa layer it was converted to aldosterone. Stachenko and Giroud (1959) confirmed these results using bovine adrenal tissue. Incubation of zona glomerulosa slices with labeled progesterone or corticosterone resulted in aldosterone secretion but addition of progesterone to either fasciculata or reticularis slices caused synthesis of hydrocortisone. These results are evidence for the specific presence of 18-oxygenating enzymes in the zona glomerulosa, 17α-hydroxylating and 11- and 21-hydroxylase activities in both the zonae fasciculata and reticularis.

1. Blood Transportation of Adrenal Steroids

In plasma, cortisol and corticosterone exist either free (biologically active) or bound to plasma proteins (biologically inactive). Slaunwhite et al. (1962) presented evidence indicating that only the freely diffusible or unbound portion of circulating cortisol is available to target tissues.

The major corticoid binding protein is transcortin or corticosteroid-binding globulin (CBG). This is a glycoprotein synthesized by the liver and has one cortisol-binding site per protein molecule. Under normal conditions

this protein reversibly binds over 90% of plasma cortisol. Albumin, the other binding protein, is contained in greater concentration in plasma than CBG but only loosely binds cortisol. However, when there is an elevation of corticoid production not only does the free moiety increase but the amount bound to albumin also increases due to saturation of the CBG binding sites.

The two binding proteins differ in that CBG has a high affinity for corticoids but a low binding capacity, and albumin a low affinity but a great binding capacity. Due to these characteristics most of the corticoids are normally bound to CBG but since the sites are easily saturated an increase in corticoid output allows greater binding to albumin. Daughaday (1967) reported that the CBG-steroid bond is augmented by the hydroxyl groups at positions 11, 17, and 21 of the steroid molecule whereas the presence of these groups suppressed binding of corticoid to albumin. The same author reported that the affinity of human CBG for cortisol is greater than for corticosterone. The 17α-hydroxyl group on cortisol may enhance affinity for human CBG.

Measurements of CBG during different conditions revealed an increase during pregnancy in humans (Daughaday, 1957; Slaunwhite and Sandberg, 1959; Doe et al., 1964; Rosenthal and Slaunwhite, 1969), followed by a decrease to

levels characteristic of nonpregnant women by two weeks postpartum. Gala and Westphal (1965) found no difference in CBG concentration between pregnant and adult virgin rats, nor did estrogen influence CBG concentration in blood. However, Mills et al. (1960) observed increased CBG concentration and cortisol half-life in women treated with estrogen.

In cattle (Krulik and Svobodova, 1969), and in sheep and cattle (Linder, 1964) found that corticosteroid-binding protein increased with age but estrogen had no effect on the CBG concentration. Gala and Westphal (1965) demonstrated that CBG concentration in blood was significantly greater in adult than in immature rats and that female rats had twice as much CBG as male rats by 48 days of age. Patterson and Hills (1967) found no significant difference in CBG concentration in pregnant sheep relative to nonpregnant controls.

In guinea pigs Gala and Westphal (1967) and Rosenthal and Slaunwhite (1969) reported increased CBG concentration in the latter stages of pregnancy relative to nonpregnant controls. Gala and Westphal (1967) also observed a significant elevation of CBG during late pregnancy in the rabbit which declined during early lactation. Floroni and Buyske (1961) reported that dog plasma contained as much CBG as human plasma. Seal and Doe (1963) observed only slight elevation during pregnancy.

2. Degradation of Cortisol and Corticosterone

The liver is the main site of degradation of cortisol and corticosterone. Products of steroid degradation are thought to have less biological activity than the parent compound. The first change that occurs in the liver is oxidation of the C-11 hydroxyl to a ketone group, thereby converting cortisol and corticosterone to cortisone and 11-deoxycorticosterone respectively. Enzyme systems which catalyze this reaction are present in peripheral tissues but predominate in the liver. Di- or triphosphopyridine nucleotides are cofactors in this reaction (Rosenfeld et al., 1967). Degradation of the double bond between C-4 and C-5 follows, producing tetrahydro compounds. Thereafter reduction of the C-20 ketone group results in formation of cortols and cortolones. These metabolites are conjugated to glucuronic acid or sulfuric acid and excreted via the kidney without participating in further biochemical transformation. There is also a minor excretion of the native hormones themselves.

E. Control of Adrenal Function

1. Hypothalamus

The hypothalamus is a small area of the base of the brain located around the third ventricle immediately behind the optic chiasma. It regulates secretion of the anterior pituitary hormones and also controls appetite, body temperature and water intake.

The pituitary gland is located in close proximity to the hypothalamus. The hypothalamus unites with the pituitary stalk at the median eminence. There is a direct neural connection between the hypothalamus and the posterior pituitary gland. Axons of the nerve cells of the supra-optic and paraventricular nuclei form a nerve tract called the hypothalmohypophyseal tract. Hormones of the posterior pituitary gland are secreted in these nuclei and transported via axons to be stored in the posterior pituitary until secreted.

Unlike the neurohypophysis there is no neural connections between the anterior pituitary and the hypothalamus. Rather, communication is via the pituitary venous portal system. This system is composed of well developed capillaries (primary plexus) in the region of the median eminence where they come in contact with terminals of hypothalamic nerve fibers. Blood flows from the primary plexus down the pituitary stalk and is distributed throughout the anterior pituitary by capillaries of the secondary plexus. Release of adenohypophysial hormones is under control of substances of hypothalamic origin which stimulate or inhibit hormone release. They are transported from the median eminence by the hypophyseal portal system to the adenohypophysis to regulate secretion of pituitary hormones. The current concept is that at least one releasing

factor exists for each hormone of the adenohypophysis except prolactin which has an inhibiting factor.

2. Corticotrophin Releasing Factor

Corticotrophin releasing factor (CRF) was the first releasing hormone demonstrated to be present in hypothalmic tissues. Saffran et al. (1955) by means of in vitro incubation of anterior pituitary tissue observed an increase in ACTH activity in the medium following addition of hypothalamic extract or cerebral cortical extract. The substance present in these tissues that triggered ACTH production was provisionally named Corticotrophin Releasing Factor. In 1957 Guillemin et al. demonstrated that hypothalamic extracts of rat, ovine or bovine origin are active in releasing ACTH from the pituitary both in vitro and in vivo. CRF activity was also demonstrated in posterior pituitary extracts and in a polypeptide preparation "Substance P" of intestinal origin. Royce and Sayers (1958) observed that rats bearing hypothalamic lesions would release ACTH following administration of hypothalamic extracts.

Although a specific releasing factor for ACTH secretion was postulated, other evidence suggested vasopressin as the active material. McCann and Brobeck (1954) observed that destruction of the supraoptic nucleus resulted in diabetes insipidus and decreased secretion of ACTH as

measured by adrenal ascorbic acid depletion. With large doses of pitressin ACTH secretion was augmented. They suggested that the supraoptic nucleus, by means of its antidiuretic hormone, was regulating ACTH release. Saffran et al. (1955) also observed CRF activity in posterior pituitary extracts but they suggested that CRF from the neurophypophysis was distinct from vasopressin. McCann (1957) demonstrated that pitressin would evoke ACTH release in hypothalamic lesioned rats. However, Guillemin et al. (1957) observed that a small dose of crude CRF would induce ACTH release in rats with hypothalamic lesions whereas only very large doses of lysine-vasopressin would evoke ACTH release.

In 1964 Guillemin reported the presence of two types of molecules in the hypothalamus capable of releasing ACTH; α-CRF, a substance closely related to α-MSH, and β-CRF, a substance closely related to vasopressin. Royce and Sayers (1958) suggested CRF to be a peptide since it is inactivated by pepsin and Ramirez and McCann (1964) reported its dissimilarity from vasopressin and oxytocin since it is not inactivated by thioglycollate. However, the complete structure is still unknown.

Recent evidence of CRF activity is reported by Porter et al. (1967) who observed that purified CRF given to rats bearing lesions in the median eminence resulted in a five-fold increase in plasma corticosterone within 20 minutes

after infusion. But the same material given to hypophysectomized rats had no effect on plasma corticosterone. Hiroshige and Sakakura (1971) observed in rats a marked CRF circadian variation which was positively correlated with plasma corticosterone being very low at 8:00 AM and at maximum level at 6:00 to 9:00 PM.

F. Stimulation of Adrenal Cortical Secretions

A variety of stimuli has been demonstrated to increase secretion and release of adrenal corticoids. These stimuli include: cold, heat, mating, ACTH, parturition and suckling. For the purpose of this thesis, discussion will be limited to the suckling or milking stimulus and parturition.

Formerly, it was thought withdrawal of milk to be the primary factor maintaining lactation. Involution of the nonmilked mammary gland was attributed to increased mammary alveolar pressure. But Selye (1934) observed that removal of the suckling stimulus was a greater factor than nonremoval of secretion in causing mammary involution in rats. Ingelbrecht (1935) demonstrated that lactation waned in rats having spinal cord lesion when suckling was restricted to teats caudal to the lesion but lactation continued normally when teats cranial to the lesion were suckled. Similar results were obtained by Eayrs and Baddley (1956) who concluded that the pathway of the

suckling stimulus to the central nervous system was through the dorsal roots of the spinal cord. In contrast, Grosvenor (1964) found that if spinal cord section was performed in rats at mid-lactation when the suckling stimulus was strongest, lactation continued, provided oxytocin was given to insure milk let down. Folley (1947) observed rapid mammary involution in rats following weaning while intensive nursing delayed involution. Presumably the suckling stimulus exerts its effects by inactivating or preventing the synthesis or release of prolactin inhibiting factor in the hypothalamus. Selye and McKeown (1934), Nicoll and Meites (1959) and Tucker and Reece (1963) reported maintenance of lactation beyond the time of normal weaning by replacing pups to maintain an intense suckling stimulus.

But the suckling stimulus per se is not required in all species. Linzell (1963) transplanted the mammary gland to the neck of a goat and observed continued secretion in the absence of neural connections. Tverskoi (1960) observed essentially normal milk yields in goats following denervation of the udder and exogenous oxytocin was not required. Similarly, Denamur and Martinet (1960) reported that spinal cord section or mammary gland denervation did not cause lactational deficiencies in sheep and goats regardless of oxytocin treatment. Presumably the secretion of galactopoietic complex by the anterior

pituitary is under humoral control. In the rabbit Mena and Beyer (1963) observed that spinal cord section depressed lactation although oxytocin was administered. However, application of prolactin or ACTH would correct the deficiency. Beyer et al. (1962) reported that section of the spinal cord resulted in failure of lactation in the cat and oxytocin injections did not correct lactational deficiency. Therefore it would seem that the importance of the milking stimulus in maintaining lactation is species dependent.

Based on thymus involution during periods of intense suckling in rats, Gregoire (1947) suggested that stimuli generated by suckling caused the release of ACTH. This was supported by Denamur et al. (1965) who observed a decrease in pituitary ACTH in sheep and goats at 30 minutes postmilking. Voogt et al. (1969) demonstrated that 0.5 hour of suckling following 12 hours nonsuckling resulted in 60% decline in pituitary ACTH and four-fold increase in plasma corticosterone. When suckling time was increased to three hours following 12 hours nonsuckling pituitary ACTH increased 85%. They concluded that the suckling stimulated pituitary ACTH synthesis and release which in turn evoked increased secretion of corticosterone from the adrenals. Tucker and Meites (1965) by means of the milking stimulus applied twice daily, initiated lactation in eight but not three months

pregnant heifers. Production was 3-lb., 12-lb., 20-lb., for the 3rd, 6th and 10th days of application of the milking stimulus. They suggested that prolactin and ACTH release was stimulated causing initiation of lactation. Similarly, Cowie et al. (1967) observed that twice daily milking of intact or ovariectomized virgin goats resulted in mammary growth and initiation of lactation but prior pituitary stalk transection prevented this response. Russian workers reported that when udders of sows or heifers are massaged before and during first pregnancy subsequent lactational performance was increased (cited in: Cowie et al., 1967). Bruce (1961) observed that by continually replacing litters, lactation can be maintained in rats for 10-12 months. Ota and Yokoyama (1965) demonstrated that use of foster litters on rats from which litters were previously removed, restored lactation but restoration became more difficult as the interval between removal of original litters and replacement of foster litters was increased.

In cows, Wagner (1969) and Wagner and Oxenreider (1971) observed a significant increase in plasma corticoid 5-10 minutes after the start of milking. A positive relationship between corticoids and oxytocin release was suggested since administration of oxytocin (10 International Units) resulted in an elevation of plasma corticoid in nonlactating cows. Paape et al. (1971) observed a

significant elevation of plasma corticoid in normal-milked and over-milked cows relative to nonmilked controls. The increase in corticoids was greatest in the over-milked cows. By 90 minutes postmilking plasma corticoids declined to premilking values.

The practical importance of the milking stimulus is evident from the fact that the more often cows are milked the greater milk production.

G. Glucocorticoid Control of the Mammary Gland

The role of exogenous corticoids in initiating and maintaining lactation has been of great interest. Cowie and Folley (1947) reported the maintenance of normal lactation in adrenalectomized rats with deoxycorticosterone (3 mg/day) injected from the 4th day of lactation. Adrenalectomy of pregnant rats did not prevent normal delivery of the young but initiation of lactation did not occur unless exogenous glucocorticoid was administered (Turner, 1966). Davis and Liu (1969) restored normal milk secretion in adrenalectomized and adrenalectomized-ovariectomized rats. These observations suggest that glucocorticoids are essential for lactogenesis in the rat. Lyons (1958) demonstrated that prolactin plus glucocorticoid constituted the minimal hormone requirement for lactation in hypophysectomized rats. Using a high dose of hydrocortisone acetate Talwalker *et al.* (1961) induced

mammary secretion in pregnant rabbits while corticosterone or hydrocortisone was effective in pregnant rats. Meites and Hopkins (1963) obtained similar results in mid-pregnant rabbits using cortisol acetate and prolactin. These results imply that either or both of these hormones may be rate limiting to initiation of lactation during pregnancy.

Injection of corticosterone into pregnant rats by Kumaresan et al. (1967) resulted in 52% increase in mammary gland ribonucleic acid (RNA) and 23% increase in deoxyribonucleic acid (DNA) relative to 20 day pregnant controls. Ferreri and Griffith (1969) reported that hydrocortisone had no effect on DNA and only increased mammary RNA in rats when it was injected during late pregnancy. Injection of hydrocortisone during mid-pregnancy failed to induce lactation as measured by the appearance of casein-like protein or increased ribonucleic acid in the mammary gland (Davis and Liu, 1969). They concluded that sub-optimal secretion of adrenal steroids was not responsible for the absence of milk secretion during pregnancy in the rat.

Thatcher (1968) reported increased lactational performance of intensely suckled rats receiving any combination of hydrocortisone acetate and other adenohypophyseal hormone. Similarly, Thatcher and Tucker (1970) observed that anterior pituitary hormones plus cortisol acetate

administered to lactating rats during prolonged suckling improved lactation relative to rats treated with only pituitary hormones. The authors concluded that adrenal corticoids may be rate limiting to lactation in the rat. Ben-David and Sullivan (1970) injected intact virgin rats with tranquilizing drugs capable of releasing prolactin and observed mammary growth and secretion. However, secretion did not occur in similarly treated adrenalectomized rats unless exogenous corticoid was provided.

Tucker and Meites (1965) initiated lactation in early, mid- and late-pregnant heifers with a synthetic glucocorticoid. The degree of milk secretion induced, seemed to depend on the stage of pregnancy during which the glucocorticoid was administered as more milk was produced from the late pregnant heifers exposed to corticoid for a shorter time. Delouis and Denamur (1967) were able to induce mammary secretion with glucocorticoid in pregnant ewes only if they were pregnant for a minimum of 100 days. They suggested that the lactogenic response seemed to be negligible before 100 days in this species.

In vitro techniques have also supported evidence for the requirement of glucocorticoid to initiate lactation. Lockwood et al. (1966) reported maximum stimulation of α-lactalbumin and β-lactoglobulin in pregnant mouse mammary gland incubated with hydrocortisone, prolactin and insulin. Similarly, Elias (1969) observed mammary

secretion in pregnant mouse mammary tissue incubated with hydrocortisone and insulin. Hydrocortisone alone, however, had no effect on secretion.

Cowie and Folley (1961) suggested three possible roles for glucocorticoids in lactation: (1) they maintain alveolar cells in a state responsive to the galactopoietic complex; (2) they maintain the necessary levels of milk precursors in the blood; and (3) the might be involved in lipogenesis. Folley and Greenbaun (1948) observed increased mammary gland arginase during early lactation, but this enzyme and milk production declined in adrenalectomized rats relative to sham operated controls. The authors hypothesized that adrenal cortical hormones increased arginase causing increased deaminized arginine residues for synthesis of milk.

Although the administration of glucocorticoid initiates lactation in most species and maintains it beyond normal weaning in rats, it seems to depress established lactation in cows. Cotes et al. (1949) reported that administration of ACTH depressed milk yields in cows relative to pre-injected levels. Similarly, Flux et al. (1954) observed that injection of 100 or 200 International Units of ACTH depressed milk yields in lactating cows relative to saline-treated controls. However, an increase in fat and milk proteins was observed. Shaw et al. (1955) also reported that injection of (200 IU) ACTH to lactating

cows resulted in decreased milk yield and increased fat and blood glucose concentrations relative to levels prior to injection. Brush (1960) demonstrated that injections of 160 (IU) of ACTH to lactating cows resulted in elevation of plasma corticoids and a decline in milk yield. More recently, Braun et al. (1970) observed that administration of glucocorticoid to lactating cows at peak production decreased milk yields and increased blood glucose, relative to nontreated controls. All these investigators observed return of normal milk yields within three to seven days after corticoids were withdrawn.

In contrast to these results, Roy (1947) reported that administration of ACTH to cows during the declining phase of their lactation augmented milk production, and had no effect on milk composition. ACTH plus prolactin resulted in a better response. Lind (1969) observed that injection of small doses of flumethazone to lactating cows increased milk yields. Larger doses, however, were inhibitory to both production and composition of milk.

H. Glucocorticoid Involvement in Parturition

Endocrine factors which cause parturition and initiate lactation remain obscure. Some investigators attribute the stimulus for parturition and initiation of lactation to the fetus while others maintain that only conditions in the dam are involved. One theory contends that high

levels of estrogen and progesterone during pregnancy render the mammary gland refractory to prolactin and glucocorticoid. High estrogen concentration is thought to increase CBG which binds more corticoid thus reducing the amount that is free or biologically active. Preceding parturition progesterone declines and estrogen becomes dominant and stimulates prolactin release. Decreased estrogen concentration at the onset of parturition results in decreased CBG and increased free corticoid which precipitates parturition. The initiation of lactation at parturition is due to the effect of increased availability of prolactin and glucocorticoid.

The above hypothesis becomes questionable when applied to ruminants if one considers the observations of Patterson and Hills (1967) and Krulik and Svobodova (1969) who reported no significant increase in CBG concentration in pregnant sheep and cattle.

Reports on plasma corticoid levels during pregnancy and at parturition are controversial and indicate species variation. Robertson and Mixner (1956) reported greater concentration of plasma corticoid in six-week-prepartum cattle relative to nonpregnant controls. Patterson (1957) observed significant plasma corticoid elevation in the cow during the last ten days of pregnancy which declined at parturition and remained low thereafter. Brush (1958) demonstrated that 17-hydroxycorticoids were slightly

increased in the cow during late pregnancy (33-0 days prepartum) relative to the early postpartum period (0-99 days). However, Adams and Wagner (1970) observed a significant increase of plasma corticoid concentration in cows four to one day prepartum which reached a maximum at parturition and declined thereafter. Heitzman (1970) reported increased levels of corticosteroids in the cow during the last 15 days of pregnancy and for a short period following parturition. Similarly, Arije et al. (1971) reported increased serum corticoid concentration in beef cows at parturition.

Poulton and Reece (1957) used "adrenal cholestrol depletion" as a measure of adrenal cortical activity and reported a decline in adrenal cholesterol in rats at parturition and during the early postpartum period. Anderson and Sperry (1937) and Anderson and Turner (1962) also reported a reduction in adrenal ascorbic acid and cholesterol in rats during pregnancy and early lactation. Gala and Westphal (1965) observed an increase in serum corticosterone during late pregnancy (20 days) in rats relative to early and mid-pregnancy values. Voogt et al. (1969) reported increased plasma corticosterone in rats at parturition relative to prepartum levels.

Reports of Patterson and Hills (1967) indicated that plasma corticoid did not change during the first 120 days of pregnancy in the sheep but in the last two weeks

preceding parturition there was a significant decline in plasma corticoid. These authors suggested that during this period of pregnancy there was an increase in the fetal adrenal secretory activity. Saba (1965) also reported a substantial decline in blood cortisol prior to parturition in the sheep. However, the mouse (Gala and Westphal, 1967; Brain and Nowell, 1970) and the guinea pig (Gala and Westphal, 1967) are like the rat in having elevated plasma corticoid during late pregnancy. In pregnant women Gemzell (1953) observed increased levels of 17-hydroxy-corticosteroids which maximized at parturition then declined thereafter.

1. Glucocorticoid Induction of Parturition

Within the last five years a number of investigators have reported successful induction of parturition in different species by using synthetic glucocorticoids. The acceptance of such a technique especially in the dairy industry would allow farm personnel to space parturition at the most convenient time suitable to the farm enterprise.

Adams (1969) demonstrated that 20 mg dexamethazone administered to cattle during the last month of pregnancy resulted in parturition within 48 hours. However, with 40 mg dosage parturition was not induced. Mossman and Conrad (1969) observed that *in vitro* incubation of pregnant

mouse or human uterine strips in low concentration of cortisol or prednisolone resulted in increased contractile activity of the tissues, but high glucocorticoid concentrations had suppressive effects. In 1969 Adams and Wagner reported that glucocorticoids successfully induced parturition in cattle, sheep and rabbits. Fylling (1970) induced parturition in ewes with 6-10 mg dexamethazone administered for four days. Parturition was preceded by a decline in plasma progesterone. When 4 mg of dexamethazone was used it had no effect on parturition or progesterone concentration. He suggested that corticoids initiated parturition by having a suppressive effect on progesterone.

Several investigators have involved the fetus in the mechanism initiating parturition. Milkovic and Milkovic (1962) reported that the adrenals of fetal rats (21-22 days) excreted as much corticosterone as adrenals of mature animals. These same authors (1963) also observed that corticosterone in the late pregnant rat decreased 50% following adrenalectomy. They suggested that maternal corticosterone concentration was maintained from fetal secretion. Dassler (1967) reported an increase in urinary corticoid excretion during late pregnancy in women, but this was diminished if fetal death had occurred. Kamoun et al. (1968) using adrenalectomized rats suggested that from the 18th day of pregnancy there was transplacental

transfer of corticosterone from the fetus to the maternal circulation (cited in: Denamur, 1971). Although Basset and Thorburn (1969) observed increased corticoid concentration in ovine fetuses just prior to parturition there was no concomitant rise in maternal plasma corticoids.

Denamur and Martinet (1961) observed that hypophysectomy of the sheep between the 50th and 134th days of pregnancy did not affect normal parturition. Drost and Holm (1968) and Drost (1969) reported that hypophysectomy or adrenalectomy of fetal lamb prolonged gestation. Similarly, Liggins et al. (1966 and 1968) and Liggins (1968) demonstrated that destruction of the pituitary of the fetal lamb in utero resulted in indefinite prolongation of pregnancy. However, after ten days prolongation administration of ACTH for six days to the fetus resulted in parturition. Hypophysectomy of one member of triplets or twins did not suspend parturition relative to sham operated controls. Holm et al. (1961) observed that postmature Holstein calves delivered by caesarean section display adrenal insufficiency. Both adrenal and adenohypophysis were underdeveloped relative to normal end of term controls. Similarly, Kennedy et al. (1957) observed that adenohypophyseal aplasia in fetal Guernsey calves prlonged gestation to an average of 401 days and the fetal adrenals at birth showed signs of atrophy. The gestation period in women with Addisons disease is also reported by

Osler (1962) to be increased by approximately 13 days relative to normal pregnant women. On the other hand, Van Rensburg (1965) reported that the fetuses from habitually aborting Angora goats had hyperplastic adrenals and atrophied thymus glands. These evidences imply that the fetal pituitary-adrenal axis plays a role in initiating parturition.

Liggins (1969) could not successfully induce parturition in pregnant ewes injected with dexamethazone (4 mg/day) towards the end of pregnancy, but this dosage was adequate if infused into the fetus. He could also induce parturition by injecting the fetus with cortisol or ACTH but not other pituitary hormones. Increases in fetal lamb corticosteroid concentration towards the end of pregnancy have been reported by Jones et al. (1964) and Alexander et al. (1969). Abramovich and Wade (1969) suggested that increased corticoid levels in human fetuses may not be due to synthesis by fetal adrenals as in this species transplacental transfer of maternal corticoid is possible.

In general, it is the consensus of opinion of many investigators that glucocorticoids are needed for initiating parturition and lactation but the mechanisms of action are still to be elucidated.

MATERIALS AND METHODS--GENERAL

A. Experimental Animals

1. Rats

Primiparous Sprague-Dawley rats were purchased from Spartan Research Laboratories, Haslett, Michigan. Upon arrival at our laboratory they were housed under controlled temperature (21°C) and lighting (14 hours light daily) with feed (Wayne Lab - Blox, Allied Mills, Inc., Chicago, Ill.) and water provided ad libitum. They were cohabited with Sprague-Dawley males and when diagnosed pregnant were housed in individual cages.

All rats were sacrificed by decapitation. Trunk blood was collected in 12x75 mm tubes containing approximately 0.5 mg ethylenediaminetetraacetic acid. Blood plasma was obtained by centrifugation (2500 xg at 4°C) then stored at -20°C until assayed for corticosterone by a fluorometric procedure. Depending on experimental design, adrenals, mammary glands and ovaries were removed, trimmed and weighed at autopsy.

2. Cows

Lactating Holstein cows and pregnant heifers maintained in the Michigan State University herd were utilized for

these studies. Blood was collected from an indwelling jugular cannula, allowed to clot at 4°C for 24 hours after which serum was obtained by centrifugation (5000 xg at 4°C). Jugular blood (40 ml) was collected from heifers via venipuncture and placed in tubes containing 31.7 mg oxalic acid. The plasma obtained by centrifugation (5000 xg at 4°C) was transferred to tubes containing 27.8 mg $CaCl_2$ to precipitate clot formation. After two days at 4°C samples were centrifuged to remove fibrin clots and obtain serum. Serum samples were stored frozen until assayed for total corticoids by competitive protein binding.

B. Corticoid Assays

1. Fluorometric Assay of Plasma and Adrenal Corticosterone

The fluorometric method of Silber et al. (1958) as modified by Thatcher (1968) was used to measure corticosterone in rat plasma. The procedure of Moncloa et al. (1959) was followed to measure adrenal corticosterone in rats.

a. Plasma Corticosterone Procedure

Prior to the start of each assay all glassware was "special" washed (Appendix 1). Duplicate samples of 0.5 ml of plasma were pipetted into 15 ml Pyrex Brand teflon-lined screw-cap tubes (Scientific Prod., Evanston, Ill.). Volume was adjusted to one ml with double glass

distilled water. A reagent blank consisting of one ml of double glass distilled water was assayed with each set of samples and served as a correction for background fluorescence.

Six ml of nanograde or redistilled reagent grade dichloromethane (Mallinckrodt Chemical Works, St. Louis, Mo.) was pipetted into each tube. Corticosterone was extracted by gentle rotation of tubes for three minutes. Extraction tubes were then centrifuged for three minutes at 1651 xg and the upper steroid hormone-free aqueous layer was aspirated and discarded. The steroid extract was partially purified by vigorous extraction with 0.75 ml of cold 0.1N NaOH for 30 seconds, followed by centrifugation at 1651 xg for three minutes. The upper aqueous NaOH steroid-free layer was discarded. Four ml of the dichloromethane extract was transferred to a clean 15 ml Pyrex Brand teflon-lined screw-cap centrifuge tube and four ml fluorescing reagent (Appendix 2) added. Corticosterone was extracted into the fluorescing reagent by vigorously shaking the tubes for one minute. The mixture was then centrifuged for two minutes at 1651 xg after which the dichloromethane layer was discarded. The reagent-hormone mixture was allowed to develop for 15 minutes before being read on a Turner model III fluorometer (Turner Associates, Palo Alto, California). The fluorometer was equipped with a primary filter providing narrow band

exitation light at 470 mμ and a secondary filter, #2A-12 (Turner Associates) providing emission at 530 mμ.

Duplicate standard curves of 0.02, 0.04, 0.08 and 1.6 μg corticosterone were prepared from a stock solution of 10 μg/ml corticosterone in ethanol and run with each assay. Four ml of fluorescing reagent was added to the standard tubes and corticosterone extracted into the reagent by shaking vigorously for one minute. Fluorescence was allowed to develop for 15 minutes then measured as described above. Plasma corticosterone concentrations were calculated from a linear regression equation based on the fluorescence of the corticosterone standards. Standards and plasma samples were corrected for background fluorescence.

Preliminary assays utilizing Corticosterone-1, 2-^{3}H (40-50 Ci/mM) revealed that this procedure routinely extracted almost 100% of the tracer steroid. Therefore, no tracer steroid was added in subsequent assays.

b. Adrenal Corticosterone Procedure

Adrenal glands were thawed, transferred to a glass grinding vessel and homogenized in four ml 0.85% NaCl in 20% ethanol (homogenizing fluid). The homogenate was transferred to a 20 ml test tube and diluted to ten ml with homogenizing fluid. Duplicate aliquots (0.5 ml) of the homogenate were pipetted into 15 ml centrifuge tubes

with teflon-lined screw caps and adjusted to one ml volume with homogenizing fluid. One ml homogenizing fluid assayed with each set of unknowns served as a correction for background fluorescence.

The contents of each tube was vigorously extracted for one minute, with five ml nanograde petroleum ether (Mallinckrodt Chemical Works, St. Louis, Mo.) then centrifuged at 1651 xg for three minutes. The upper steroid-free petroleum layer was aspirated and discarded. Thereafter six ml of dichloromethane was added and the procedure followed as outlined for plasma corticosterone.

2. Protein Binding Assay

Corticoids in bovine serum were quantitated by competitive protein binding analyses (Murphy, 1967). This assay depends upon the following principle. If a stable steroid is introduced into a system containing radioactive steroid and its binding protein, the stable steroid will displace the radioactive steroid in proportion to its concentration. Comparing displacement of radioactive steroid by known quantities of stable steroid (standards) and steroid in assay samples allows estimation of concentration of steroid in biological samples.

a. Preparation of Binding Protein

Commercial dog plasma (Colorado Serum Co. Labs., Denver, Colorado) was used as the source of binding

protein. Dog plasma was stored in five ml quantities at -20°C until prepared for use in an assay. In preparation for assay, 2.5 ml dog plasma was diluted to 100 ml with double glass distilled water. Florisil (8 gm, 60-100 mesh; Fisher Scientific Co., Fair Lawn, N.J.) was added and the contents stirred on a magnetic mixer for 15 minutes. The Florisil was precipitated by centrifugation for ten minutes at 5000 xg and the supernatant decanted and diluted to a final suitable concentration for assay. Because of variation in binding capacity of dog plasma, a suitable concentration of dog plasma for use in these assays was determined for each new shipment by running standard curves with varying concentrations of dog plasma (0.625, 1.0 and 1.25%). In all assays reported herein, 1.0% dog plasma was found most satisfactory. Cortisol-1,2-^{3}H at 20,000 cpm/ml 1% dog plasma was used as competitor. After adding labeled hormone the dog plasma was gently mixed, stored at 4°C and used for assay within seven days after preparation.

b. Serum Corticoid Extraction Procedure

Approximately 1500 cpm of cortisol-1,2-^{3}H in ethanol (49.5 Ci/mM) was placed into 20 ml extraction vials fitted with polyethylene screw caps (Rochester Scientific Co., Inc., Rochester, N.Y.) previously silated with 5% chlorotrimethylsilane (Eastman Kodak Co., Rochester, N.Y.) in toluene. To determine the number of counts added to each

vial, equal volumes of cortisol-1,2-^{3}H were placed into each of two scintillation vials, five ml of steroid scintillation fluid (Appendix 3A) added, and radioactivity counted for ten minutes in a liquid scintillation spectrophotometer (Nuclear Chicago Model, Mark 1). One ml of serum was added to the extraction vials and extracted with six ml redistilled 2, 2, 4-trimethylpentane (iso-octane) (Mallinckrodt Chemical Works, St. Louis, Mo.) by stirring on a vortex mixer (Deluxe Mixer, Scientific Prod., Evanston, Ill.) at high speed for two minutes. Using disposable pipettes, the iso-octane-progestin fraction was transferred into previously silated 12 ml conical tubes and the samples reextracted with six ml iso-octane. Thereafter samples were twice extracted with six ml of redistilled dichloromethane by stirring on a vortex mixer at moderate speed for two minutes. The dichloromethane-corticoid fraction was transferred, using disposable pipettes, to silated 12 ml conical tubes and dried under nitrogen to approximately three ml volume. To determine extraction efficiency, 500 µl of the extract was evaporated in scintillation vials, five ml of scintillation fluid (Appendix 3A) was added and the sample counted in a liquid scintillation spectrophotometer. Dilution duplicates (1000 and 500 µl) of each extract were pipetted into silated culture tubes (12x75 mm). Duplicate standard curves; 0.0, 0.1, 0.5, 1.0, 1.5, 2.0, and 5.0 ng/ml of cortisol were

prepared for each assay. Culture tubes containing unknown and standards were dried under nitrogen, transferred to an ice bath and one ml, 1% dog plasma-^{3}H-2,1-cortisol mixture added. All tubes were vortexed for 15 seconds, covered and incubated for 18-24 hours at 4°C.

Unbound ^{3}H-cortisol was removed from the reaction volume by adding one ml dextran-coated charcoal (Appendix 4). The content of each tube was mixed and approximately five minutes from the onset of adding the charcoal each tube was centrifuged at 2500 xg for 15 minutes. Supernatant (0.5 ml) was removed with an automatic diluter (Model D-1000, York Instrument Corp., New York, N.Y.) then flushed from the diluter tip with Bray's solution (Appendix 3B). Samples were counted for ten minutes in a liquid scintillation spectrophotometer. Percent of labeled hormone bound was derived by plotting the average counts of the standards as a percentage of the zero tubes. Average of standard curves is presented in Figure 1. The amount of corticoid in each sample was calculated (after correcting for recovery) by interpolation between the standards. A standard plasma sample and water blank were carried through all assays as an internal standard and background check, respectively. Water blanks were uniformly low in each assay averaging 0.06 ng/ml (n=17) and the standard plasma averaged 10.0 ± 0.2 ng/ml (n=14). A criterion for validating the assay was based on recovery of unlabeled

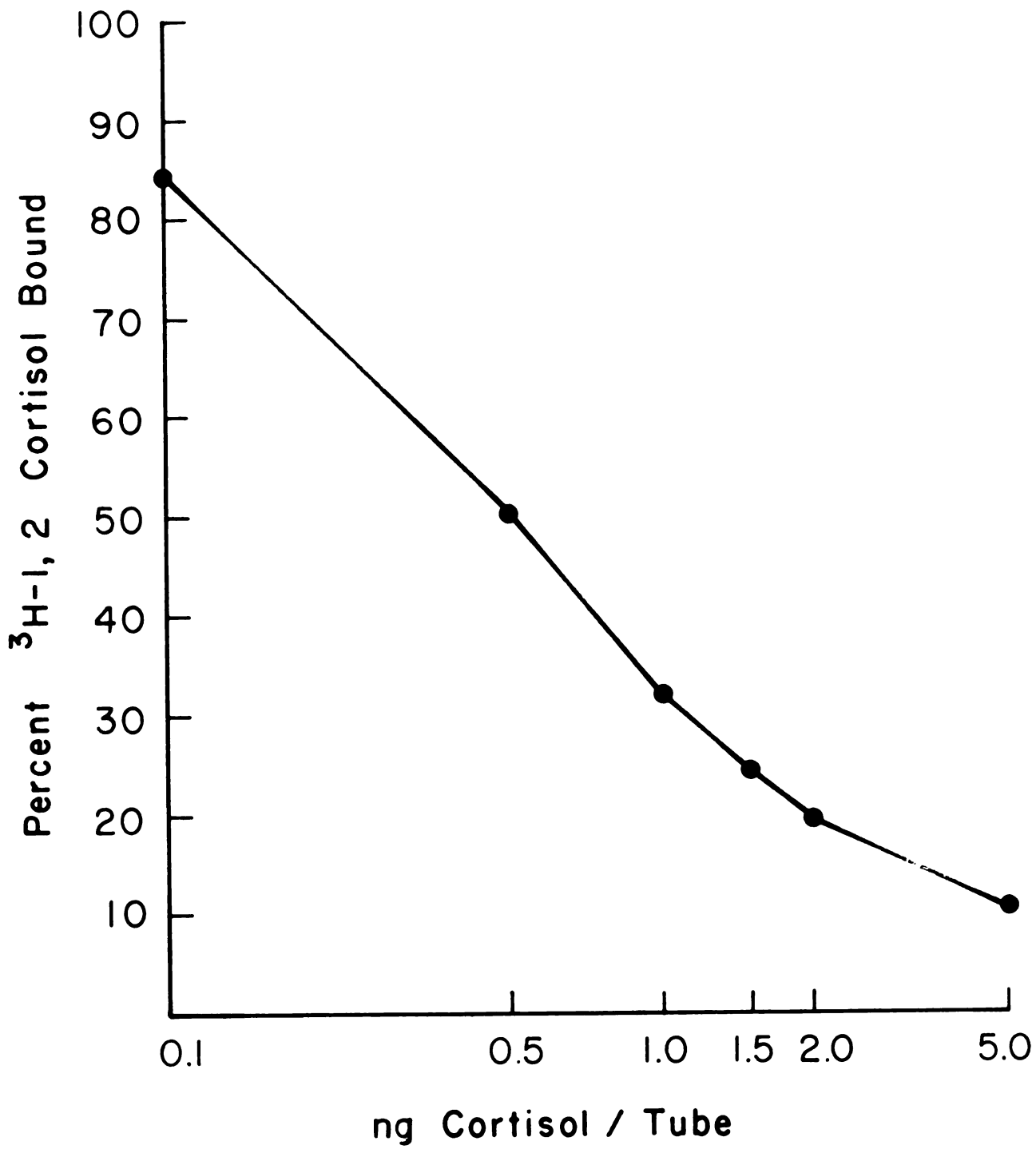


Figure 1. Standard curve. Average of 35 standard curves.

cortisol added to the standard plasma. Recovery ranged from 80% to 108% (avg 94.1 ± 3.6%, n=9).

3. Adrenal Ascorbic Acid Procedure

Trimmed adrenals were transferred to a glass homogenizing vessel containing seven ml of 2.5% metaphosphoric acid, homogenized with a teflon pestle and the suspension filtered through Whatman #1 filter paper (W. & R. Balston, Ltd., England). Adrenal homogenates were assayed for ascorbic acid by the method of Mindlin and Butler (1938).

A concentrated standard solution of 0.1 mg/ml ascorbic acid (U.S.P. C grade, California Corp., Biochem. Res., Los Angeles) was prepared from a stock solution of one mg/ml ascorbic acid dissolved in 2.5% metaphosphoric acid. One, two and three ml (of 0.1 mg/ml standard) were adjusted to 25 ml volume with 2.5% metaphosphoric acid yielding working standards of 4.0, 8.0 and 12.0 µg/ml ascorbic acid. Standards and adrenal homogenates (3 ml) were pipetted into individual 20 ml glass test tubes and five ml indophenol-acetate (Appendix 5) was added. The contents of each tube was mixed, and read at 515 mµ on a Beckman, DB spectrophotometer (Sargent and Co., Fullerton, California) against a reagent blank: five ml indophenol acetate - 3 ml 2.5% metaphosphoric acid. The spectrophotometer reading for each sample and unknown was completed within 60 seconds after addition of indophenol acetate. Adrenal ascorbic acid concentration was calculated

from a linear regression equation based on optical density of ascorbic acid standards and expressed as µg/100 mg adrenal tissue (wet weight).

C. Experimental Procedure

1. Experiment I--Influence of Acute and Chronic Suckling on Plasma Corticosterone and Adrenal Ascorbic Acid

Sixty lactating rats were used in this experiment. At least three times per week rats were handled with the objective of minimizing stress when removed from their cages for sacrifice. On the second day of lactation the thoracic teats were ligated and rats were assigned at random to a 2x3 factorial arrangement of treatments. The factorial arrangement consisted of two litter sizes and three suckling regimes. Litter size was adjusted to two or six pups on day two of lactation. On day seven of lactation litters were either (a) retained with their mother; (b) isolated for 12.5 hours, or (c) isolated for 12.0 hours then reunited with their mother for 0.5 hour of suckling. Litters in the latter group were weighed following isolation and again after the suckling period. All rats were sacrificed between 6:30 and 7:30 AM. We only used litters that had successfully suckled as evidenced by an increase in litter weight and milk in the pups' stomachs. Adrenals were weighed, homogenized in cold (4°C) 2.5% metaphosphoric acid and stored until assayed for ascorbic acid.

2. Experiment II--Adrenal Response to Pregnancy or Lactation in the Rat

In this experiment 70 pregnant or lactating rats were used. On day zero of pregnancy (first appearance of sperm in vaginal smear) rats were randomly assigned to five groups. These groups were: (1) day 11 of pregnancy, (2) day 19 of pregnancy, (3) parturition, (4) day two of lactation, and (5) day 15 of lactation. Rats in the latter two groups had their litters adjusted to six pups on day one of lactation. Rats in group three were sacrificed during delivery of the young and all others between 3:30 and 4:30 PM. Plasma and adrenals were assayed for corticosterone.

3. Experiment III--Serum Corticoid Response to Milking and Nonspecific Stimuli

Three pairs of cows in late lactation were utilized. They were housed in two separate sections of the Michigan State University dairy barn, with both members of a pair stanchioned beside each other. Experiments on two pairs were completed before the third pair was placed on experiment. This arrangement allowed each pair to be milked separately and not be influenced by exteroceptive stimuli that might be associated with milking another pair. During the experimental period the milking schedule was delayed from 3:00 to 7:00 PM to achieve a 12 hour milking interval. Therefore a four day conditioning period during which cows

were milked at 7:00 PM was included in the experimental design. During the four day conditioning period, cows of a pair were alternately milked first.

Two days preceding the experiment an indwelling jugular cannula (Vinyl IV Tubing, Clay Adams, Inc., New York, N.Y.) was inserted. Approximately 18 inches of the eight foot cannula was placed in the jugular vein, and affixed to the neck and withers with branding cement on three-inch adhesive tape. It was passed along the dorsal midline of the cow to the rear and affixed to the tail head. This arrangement facilitated collection of blood while standing at the rear of the cow.

One cow in each of two pairs was milked on day one and her pairmate milked on day two. The last pair was similarly treated on days three and four. Approximately 20 ml of blood was drawn from each cow of a pair, simultaneously on each day at ten, five and one minute before milking, at the time the milking machine was placed on the cow and at one, three, five, 15, 60, and 90 minutes after the machine was placed on the cow. During the minute preceding milking, the udder of the milked cow was washed, the vacuum pump turned on and the milking machine brought into the barn. The milking machine was applied to the teats at time zero. Each day the control cow was milked after the 90 minute sampling period.

4. Experiment IV--Pre and Postpartum Plasma Corticoid in Heifers

In this experiment ten pregnant heifers (part of another project at our laboratory) were utilized. Approximately ten days before expected calving they were transferred to calving stalls. Each heifer was bled twice weekly from 26 to six days prepartum, twice daily, (8:00 AM and 5:00 PM) from six days before to five days after parturition and at nine days postpartum.

RESULTS

Experiment I--Influence of Acute and Chronic Suckling on Plasma Corticosterone and Adrenal Ascorbic Acid

Final body, mammary and ovarian weight of rats subjected to acute or chronic suckling are shown in Table 1. Orthogonal contrast revealed no significant effect ($p > 0.05$) of litter size or acute suckling on body weight. Average body weight was 228.3 and 231.6 g for rats suckling two or six-pup litters respectively.

Average mammary weight of rats suckling six-pup litters (12.6 g) was greater ($p < 0.01$) that the comparable average of rats with two-pup litters (7.6 g). Removal of litters for 12.5 hours resulted in an accumulation of milk resulting in increased mammary weight. However, this increase was significant ($p < 0.01$) only in rats suckling six pups. Milk removal by two or six pups during 0.5 hour of suckling decreased mammary weight relative to the nonsuckled controls, but in neither case was the decrease significant ($p > 0.05$).

Altering the suckling period on day seven of lactation did not significantly ($p > 0.05$) change average ovarian weight of rats suckling either two or six pups. Therefore ovarian weight within litter size was averaged across the

Table 1. Influence of Number of Young and Suckling Stimulus on Body, Mammary and Ovarian Weight[a]

Litter size	No. of rats	Suckling period (hr)		Weight		
		Nonsuckling	Suckling	Body (g)	Mammary (g)	Ovaries (mg)
2	10	0.0	12.5	229.8±4.7	7.0±0.3	77.6±2.6
	10	12.5	0.0	222.5±6.7	8.1±0.5	72.0±3.1
	10	12.0	0.5	232.6±4.6	7.6±0.4	84.7±4.0
Av				228.3±3.1	7.6±0.2[b]	78.1±2.1[d]
6	10	0.0	12.5	221.1±3.8	8.4±0.3[c]	64.7±3.2
	10	12.5	0.0	234.8±4.8	15.8±0.7	71.6±4.0
	10	12.0	0.5	238.9±4.6	13.6±0.4	74.6±4.1
Av				231.6±3.7	12.6±0.7	70.3±2.2

[a]Values are means ± SE.

[b]Less than comparable average for six pups ($p < 0.01$).

[c]Less than 15.8 and 13.6 ($p < 0.01$).

[d]Greater than comparable average for six pups ($p < 0.01$); interaction of litter size and suckling period not significant.

suckling periods. The ovaries of rats suckling two pups averaged 78.1 mg which was significantly greater ($p < 0.05$) than the comparable average of rats suckling six pups (70.3 mg).

Restriction of litter size to two or six pups did not significantly ($p > 0.05$) influence adrenal weight by day eight of lactation (Table 2). But within the group with six-pup litters, average adrenal weight of rats from which litters were isolated for 12.5 hours (54.8 mg) was significantly less ($p < 0.05$) than that of rats subjected to 0.5 hour of suckling, following litter isolation for 12.0 hours (65.1 mg).

Plasma corticosterone concentration of rats suckling two or six pups averaged 27.5 and 39.5 µg/100 ml respectively (Table 2) and the difference between means was significant ($p < 0.05$). Isolation of two or six-pup litters for 12.5 hours reduced plasma corticosterone 17.4 and 31.3% respectively, relative to constantly suckled controls. However, only the latter value was significant ($p < 0.05$). Twelve hours of isolation followed by 0.5 hour of suckling by two or six pups increased ($p < 0.01$) plasma corticosterone 100.5 and 334.0%, respectively, relative to corresponding nonsuckled control values. Neither litter size nor acute suckling affected adrenal ascorbic acid concentration.

Table 2. Effect of Suckling on Plasma Corticosterone (PC) and Adrenal Ascorbic Acid (AAA) Concentration of Rats Suckling Two or Six Pups[a]

Litter size	No. of rats	Suckling period (hr)		Adrenal wt. (pair) (mg)	PC (µg/100 ml)	AAA (µg/100 mg)
		Nonsuckling	Suckling			
2	10	0.0	12.5	58.1±2.0	23.8±3.4	493.5±28.7
	10	12.5	0.0	56.2±1.9	19.7±1.8[c]	491.0±29.0
	10	12.0	0.5	59.7±2.6	39.4±8.8	482.6±35.4
Av				58.0±1.2	27.5±3.5[d]	489.0±17.3
6	10	0.0	12.5	61.2±2.0	29.3±3.6	495.2±30.8
	10	12.5	0.0	54.8±2.2[b]	20.2±2.7[e]	468.4±30.4
	10	12.0	0.5	65.1±2.4	70.0±14.9	440.9±28.9
Av				60.4±1.4	39.5±6.6	468.1±17.2

[a]Values are means ± SE.

[b]Less than 65.1 ($p < 0.05$).

[c]Less than 39.4 ($p < 0.01$).

[d]Less than comparable average for six-pup group ($p < 0.01$).

[e]Less than 29.3 ($p < 0.05$) and 70.0 ($p < 0.01$).

Experiment II--Adrenal Response to Pregnancy or Lactation in the Rat

Average body weight of rats pregnant 19 days, 306.3 g was greater ($p < 0.001$) than the comparable values for rats killed on day 11 of pregnancy (249.1 g) or rats killed during parturition (278.3 g), Table 3. Body weight of rats killed on day 15 of lactation averaged 287.3 g and was significantly greater ($p < 0.001$) than the average value for rats sacrificed on day two of lactation (262.1 g).

Mammary weight of rats killed on day 11 of pregnancy averaged 3.9 g, significantly less ($p < 0.001$) than the comparable average (7.5 g) for rats killed on day 19 of pregnancy (Table 3). Mammary weight of rats killed at parturition was 9.8 g which was greater ($p < 0.001$) than mean mammary weight on day 19 of pregnancy but not different from that on day two of lactation. The mammary glands from rats killed on day 15 of lactation were heaviest ($p < 0.001$). Average mammary weight during the postpartum period was significantly greater ($p < 0.001$) than comparable average during the prepartum period.

Adrenal gland weight averaged 63.5 and 68.7 mg for rats killed on day 11 of pregnancy and three days prepartum respectively (Table 4), and the difference between means was significant ($p < 0.05$). Average adrenal weight of rats killed on day 15 of lactation was greater ($p < 0.001$)

Table 3. Body and Mammary Weight of Rats[a] During Pregnancy, Parturition and Lactation

Treatment period	No. of rats	Weight (g)	
		Body[b]	Mammary[c]
Day 11 pregnancy	14	249.1±3.0	3.9±0.1
Day 19 pregnancy	14	306.3±4.7	7.5±0.3
Parturition	14	278.3±5.6[d]	9.8±0.5[d]
Day two lactation	14	262.1±3.5	9.2±0.4[d]
Day 15 lactation	14	287.3±4.4[d]	10.9±0.4

[a]Values are means ± SE.

[b]Uncorrected for weight of conceptus.

[c]Weight of six abdominal inguinal glands.

[d]Values in a column having the same superscript are not different ($p > 0.05$).

Table 4. Rat Plasma and Adrenal Corticosterone Concentration During Pregnancy, Parturition and Lactation[a]

Treatment period	Adrenal weight mg	Corticosterone concentration	
		Adrenal µg/100 mg	Plasma µg%
Day 11 pregnancy	63.5±1.2[b]	4.3±0.5	28.3±4.5
Day 19 pregnancy	68.7±1.6[c]	5.6±0.4[b]	40.3±3.2[b]
Parturition	65.9±2.5[b,c]	5.6±0.6[b]	40.9±5.5[b]
Day two lactation	63.5±1.9[b]	6.0±0.5[b]	38.2±3.1[b]
Day 15 lactation	73.2±1.4	7.6±0.8	39.6±3.6[b]

[a]Values are means ± SE for 14 rats.

[b,c]Values in a column having the same superscript are not different ($p > 0.05$).

than the comparable value for rats lactating two days. Adrenal corticosterone (Table 4) was increased ($p < 0.05$) from 4.3 µg/100 mg for rats killed on day 11 of pregnancy to 5.6 µg/100 mg for rats killed three days prepartum. There was no significant effect ($p > 0.05$) of parturition on adrenal corticosterone concentration relative to average values on day 19 of pregnancy or day two of lactation. Continually suckling six pups through to day 15 of lactation, increased ($p < 0.01$) adrenal corticosterone concentration relative to the average value for the second day of lactation with similar number of pups. Average plasma corticosterone concentration (Table 4) on day 11 of pregnancy was 28.3 µg% and this was significantly less ($p < 0.005$) than the comparable value (40.3 µg%) at day 19 of pregnancy. There was no significant change ($p > 0.05$) in plasma corticosterone concentration at parturition relative to values at day 19 of pregnancy or lactation.

Experiment III--Serum Corticoid Response to Milking and Nonspecific Stimuli

Corticoid concentration of serum from milked and nonmilked cows is shown in Figure 2. Prior to milking, serum corticoid concentration averaged 3.9 ng/ml and was increased ($p < 0.05$) to 8.1 and 11.5 ng/ml at five and 15 minutes after the start of milking, respectively. Orthogonal contrast revealed the average premilked serum

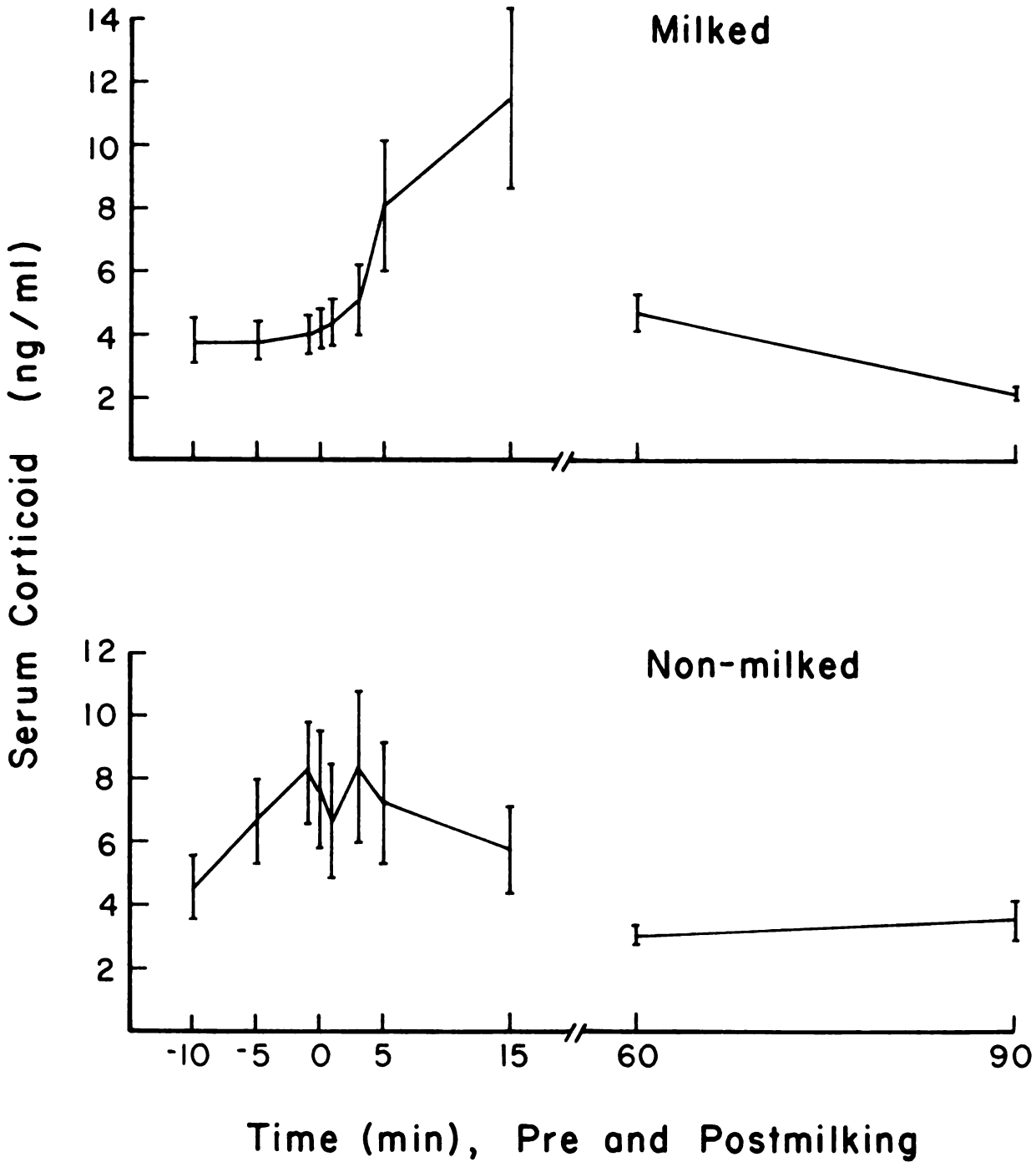


Figure 2. Serum corticoid response of cows to milking and nonspecific stimuli. Each point the mean of six observations. Vertical bars represent standard errors.

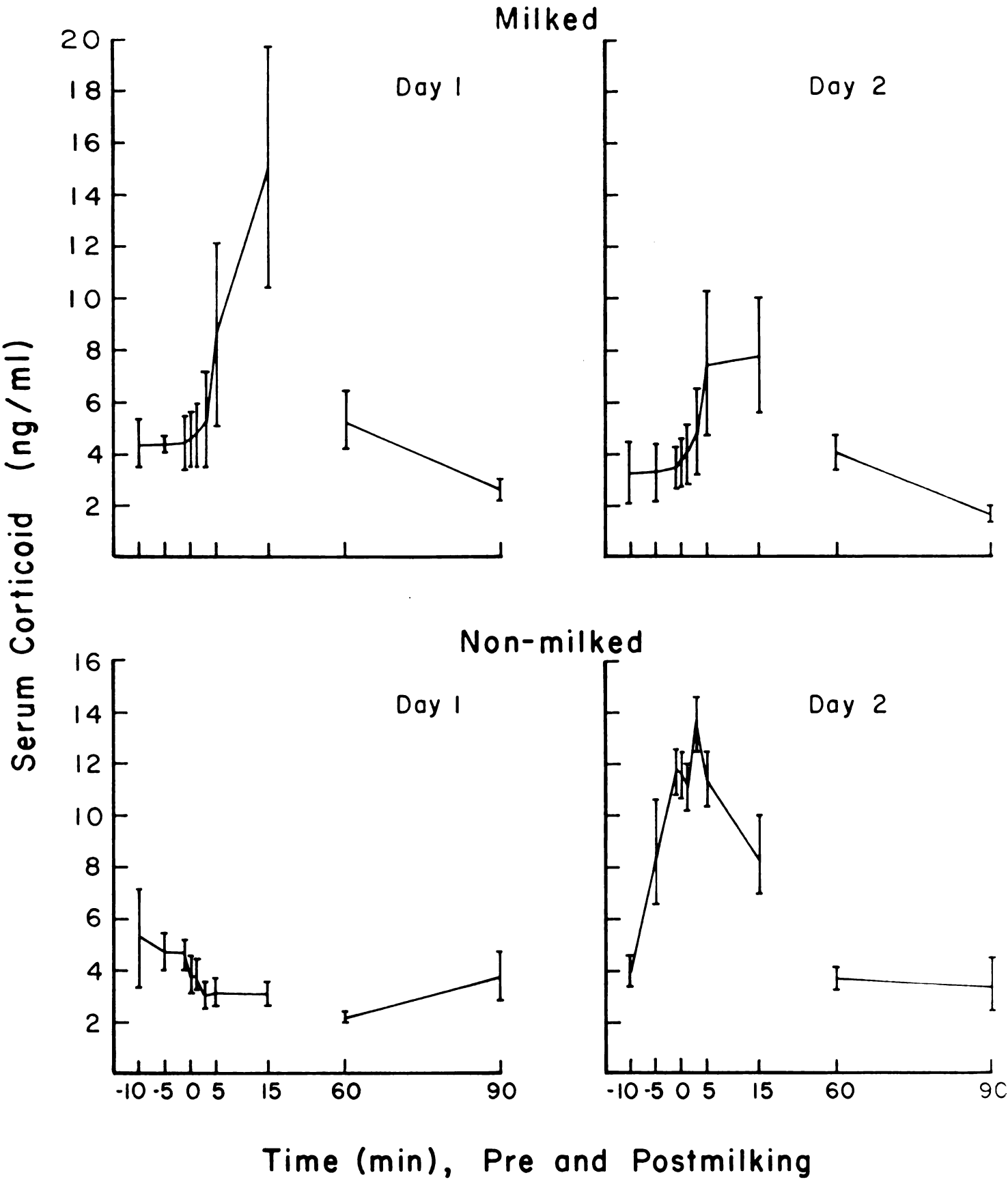
corticoid concentration to be lower ($p < 0.05$) than the postmilked average although serum corticoid concentration had declined to premilked levels by 60 minutes after milking. Serum corticoid concentration of nonmilked cows, sampled while their pairmates were milked, followed a somewhat different pattern.

Overall analysis showed a slight but nonsignificant increase in serum corticoid concentration from 4.6 ng/ml ten minutes before milking to 6.7 and 8.2 ng/ml at -5 and -1 minute respectively. Thereafter serum corticoid concentration remained high through 15 minutes postmilking but by 60 minutes after the start of milking, had decreased ($p < 0.05$) to 3.1 ng/ml, 32% lower than the comparable value at ten minutes premilking.

Effect of day or order of milking importantly influenced the nature of the corticoid response of milked and nonmilked pairmates. Accordingly, these data were replotted, according to day, on Figure 3.

With regard to milked cows on day one, serum corticoid concentration averaged 8.6 and 15.1 ng/ml at five and 15 minutes respectively after the start of milking and decreased ($p < 0.05$) thereafter. A similar increase, although of lesser magnitude, was observed on day two when serum corticoid concentration at five and 15 minutes postmilking averaged 7.5 and 7.8 ng/ml, respectively, which was significantly higher ($p < 0.05$) than the comparable

Figure 3. Serum corticoid response of cows on days one and two to milking and nonspecific stimuli. Each point the mean of three observations. Vertical bars represent standard errors.


Milked
Day 1
Day 2
Non-milked
Day 1
Day 2
Serum Corticoid (ng/ml)
20
18
16
14
12
10
8
6
4
2
-10 -5 0 5 15 60 90
Time (min), Pre and Postmilking

average ten minutes before milking (3.3 ng/ml). Failure of one cow to respond accounts for the reduced peak height and large standard error on day two.

Analysis of variance of data from nonmilked cows indicated a significant ($p < 0.01$) effect, not only of time but also of milking order or days and time by day interaction. The significant interaction resulted from failure of nonmilked cows to respond similarly on days one and two (Figure 3). On day one, serum corticoid concentration of nonmilked cows did not change ($p > 0.05$) over the sampling period. In contrast, serum from cows sampled on day two showed a significant increase ($p < 0.05$) in corticoid concentration at five minutes before milking had begun and which persisted to 15 minutes postmilking. It is important to emphasize that all nonmilked cows on day one failed to respond to treatment with increased serum corticoids while all responded on day two. In addition, serum corticoids started to increase as early as five minutes before the milking machine was brought into the barn.

Experiment IV--Pre and Postpartum Serum Corticoid Concentration in Heifers

Serum corticoid concentration averaged 5.0 ng/ml from 26 to one day before parturition, increased ($p < 0.005$) to 10.3 and 16.7 ng/ml, approximately 12 hours before and at parturition, respectively (Figure 4). By

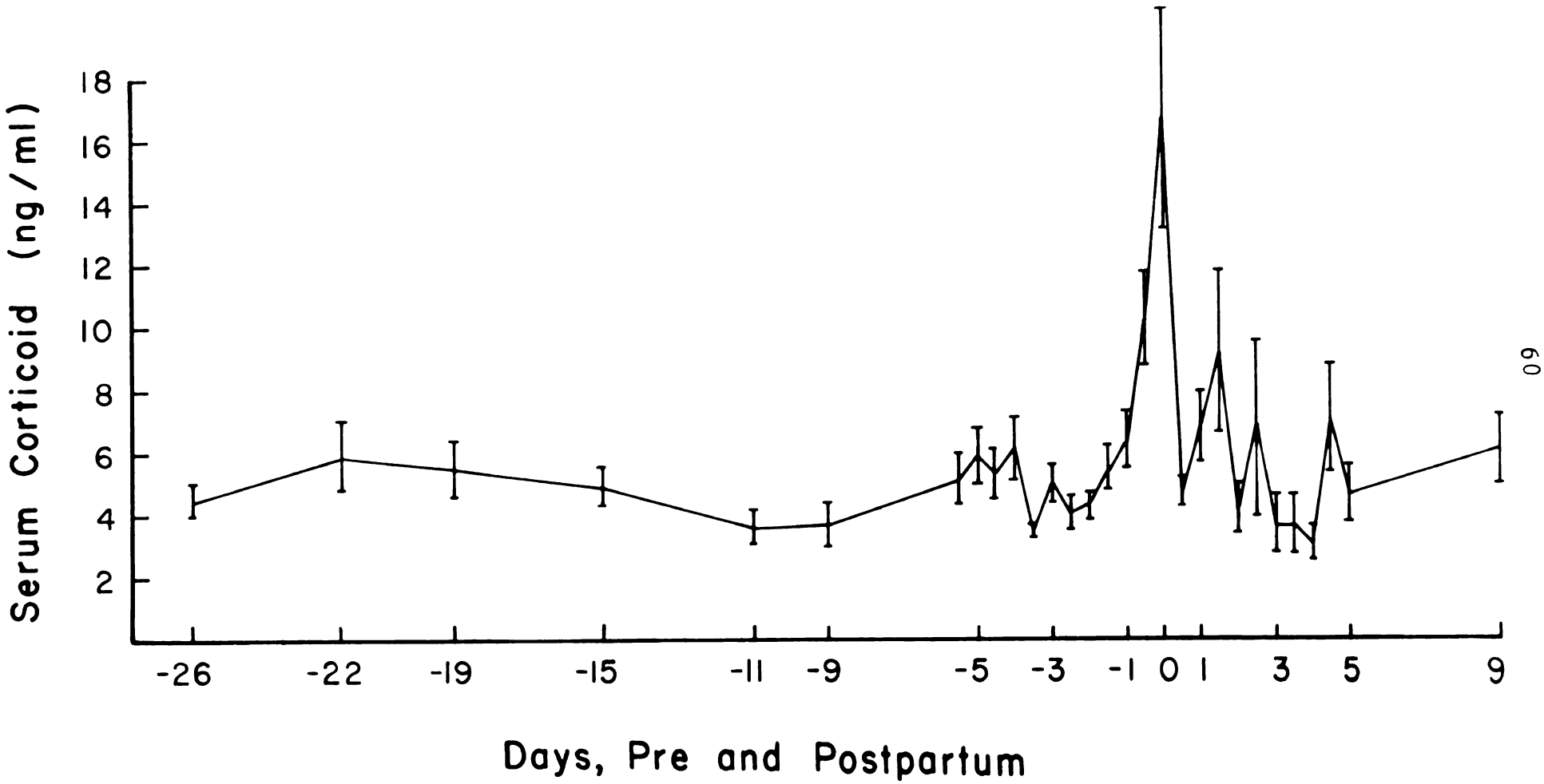


Figure 4. Corticoid concentration in serum of heifers during late pregnancy, parturition and early lactation. Vertical bars represent standard errors.

approximately 12 hours postpartum, serum corticoid concentration declined to 5.1 ng/ml; which was significantly ($p < 0.001$) lower than the mean corticoid concentration at parturition and 12 hours prepartum but not different ($p > 0.05$) than the average corticoid concentration during the period, 26 to one day prepartum. Orthogonal contrast revealed increased ($p < 0.001$) serum corticoid concentration at 36 hours postpartum relative to the average value at 12 hours after parturition. Variation in serum corticoid concentration during the first nine days postpartum was more marked than during the prepartum period. However, average serum corticoid concentration of 5.6 ± 0.5 ng/ml, postpartum, was not different ($p > 0.05$) from comparable prepartum value of 5.0 ± 0.2 ng/ml.

Although the experiment was not designed to test diurnal variation, serum corticoid concentration in samples collected at 8:00 AM and 5:00 PM from days five to one prepartum were analyzed for diurnal effects. Serum corticoid concentration of samples collected in the morning averaged 5.2 ± 0.3 ng/ml (n=49) and was not different ($p > 0.05$) from comparable afternoon values, which averaged 5.0 ± 0.4 ng/ml (n=50).

GENERAL DISCUSSION

Increased plasma and adrenal corticosterone concentration of rats killed on day 19 of pregnancy relative to comparable values for rats sacrificed on day 11 of pregnancy suggests that activity of the adrenal-pituitary axis is increased as pregnancy advances. Failure to detect changes in either plasma or adrenal corticosterone concentration at parturition relative to day 19 of pregnancy suggests that corticoid concentration reaches a peak during pregnancy, well in advance of parturition. In contrast serum corticoid concentration in cattle showed a marked increase beginning at 24 hours prepartum which reached a peak at parturition. Because the data reported here for cattle consider only the last three weeks of an approximately 282 day gestation period, corticoid changes over pregnancy cannot be assessed.

Gala and Westphal (1965) reported increased corticosterone concentration in serum from rats killed on day 20 of pregnancy relative to those killed on days eight or 14 of pregnancy. Their data did not, however, include corticosterone values for rats killed at parturition. In contrast to results presented here, Voogt *et al.* (1969) reported a significant 139% increase in plasma

corticosterone in rats at parturition, relative to that of rats killed on day 20 of pregnancy. Since data reported by the latter authors were generated from rats killed between 8:00 and 10:00 AM and data reported here were from rats killed between 3:30 and 4:30 PM involvement of diurnal changes in corticoid concentration must be considered. Critchlow et al. (1963) observed that rats provided with 12 hours darkness (6:00 PM to 6:00 AM) exhibited peak corticosterone concentration between 3:00 and 7:00 PM. Critchlow (1963) reported that rats subjected to ten hours of darkness (6:00 PM to 4:00 AM) had increased plasma and adrenal corticosterone concentration at 3:00 PM, reaching maximum concentration at 7:00 PM. McCarthy et al. (1960) also reported increased plasma corticosterone concentration in rats killed during the afternoon. Halberg et al. (1959) observed similar trends in mice. Corticosterone values in the present experiment are consistent with increased corticosterone concentration in the afternoon. At each stage of pregnancy they are approximately two-fold greater than comparable values reported by Voogt et al. (1969), but at parturition the values for the two experiments are within one ng. Therefore, the possibility must be considered that the capacity of the adrenal to respond to stimuli, is limited by the existing level of steroid production. If under these physiological conditions corticosterone synthesis and release was near

maximum it could be expected that additional stimuli associated with parturition might not result in increased corticosterone production. Other workers in our laboratory (Pratt and Convey, unpublished) killed rats between 8:00 and 9:00 AM at the same stages of pregnancy and lactation as reported here and on the day of parturition. Plasma corticosterone concentrations during pregnancy and lactation were comparable to those reported by Voogt et al. (1969) but did not increase on the day of parturition. If plasma corticosterone is increased at parturition in our colony, the increase must be transient since there was no increase in mean corticosterone concentration of rats killed on the day of parturition. The question of the response of plasma corticosterone to parturition in rats remains equivocal. Increased plasma corticosterone concentration coincident with increased mammary growth during pregnancy may reflect the metabolic regulating effect of glucocorticoids. Thatcher (1968) reported significant correlation between adrenal corticosterone content and nucleic acid contents in 16 day lactating rats.

No difference in bovine serum corticoid concentration was found in the cow between blood samples collected at 8:00 AM and 5:00 PM in the present experiment. Wagner and Oxenreider (1971) reported no difference in plasma cortisol in three nonlactating cows sampled at 30 minute

intervals during a 24 hour period. Increased corticoid concentration in serum of heifers at parturition is in agreement with results reported by Adams and Wagner (1970). They observed an increase in plasma corticoid in cows, from four to one day prepartum which peaked at parturition and declined thereafter. Shultze (1955) observed a decrease in eosinophil numbers in bovine blood at parturition relative to the prepartum period which he suggested to result from ACTH release. In contrast to these results, Brush (1958) observed no difference in plasma corticoid concentration between the last five days prepartum and parturition in cows, but following parturition plasma corticoid concentration decreased. Similarly, Saba (1965) and Patterson and Hills (1967) reported reduced plasma corticoid concentration near or at parturition in ewes. Appleby and Norymberski (1957) observed a decrease in metabolism of cortisol during the latter stages of pregnancy which may account for increased blood levels near or at parturition.

The physiological significance of increased serum corticoid concentration prior to parturition may lie in initiating parturition and lactation. Adams (1969) and Adams and Wagner (1969) successfully induced parturition in cattle, sheep and rabbits with exogenous glucocorticoid during the final stages of pregnancy. Similar results have been reported for ewes by Fylling (1970). Tucker

and Meites (1965) used a synthetic glucocorticoid to induce lactation in pregnant heifers. Using in vitro techniques, Lockwood et al. (1966) reported stimulation of milk proteins in pregnant mouse mammary tissue, incubated with hydrocortisone. Talwalker et al. (1961) induced mammary secretion in rats and rabbits by injecting hydrocortisone acetate.

The possibility that corticoid is increased near or at parturition due to stimuli associated with labor cannot be overlooked. In cattle Gilette and Holm (1963) reported increased abdominal and uterine contractions from two days prepartum to parturition. Hindson et al. (1965) observed in sheep increased frequency and amplitude in uterine activity from 12 hours before parturition. Therefore, increased serum corticoid in cows, near or at parturition, may result from stimuli associated with uterine and abdominal contractions.

Results of the present author support those of Gala and Westphal (1965) who reported that rats suckling 12 pups had greater serum corticosterone concentration than rats nursing four-pup litters. Voogt et al. (1969) reported a four-fold increase in plasma corticosterone due to 30 minutes of suckling following 12 hours nonsuckling which is in close agreement with results presented here. Based on observations of thymus involution during intense suckling in rats, Gregoire (1947) suggested that stimuli

generated by suckling promoted the release of ACTH. Denamur et al. (1965) confirmed this hypothesis when they demonstrated a marked depletion of pituitary ACTH in goats and sheep 30 minutes after milking.

Increased serum corticoid concentration in milked cows at five and 15 minutes after the start of milking confirms previous results (Wagner, 1969). Paape et al. (1971) similarly observed increased plasma corticoid concentration in normally milked and overmilked cows. Results of the present author demonstrate that serum corticoid concentration increased before the start of milking in day two nonmilked cows. This implies that exteroceptive stimuli are capable of eliciting corticoid release. Voogt et al. (1969) reported that when lactating rats were allowed to see and smell but not suckle their pups, plasma corticosterone was increased. The influence of exteroceptive stimuli on hormone release is not confined to ACTH. Van der Lee and Boot (1956) observed that housing of female mice in groups of four resulted in pseudopregnancy presumably the result of prolactin release. This effect could be prevented by housing them separately or by excising the olfactory bulbs. Alloiteau (1962) observed that separation of lactating rats from their pups at parturition prevented pseudopregnancy but when mothers were caged in groups pseudopregnancy developed. Grosvenor

(1965) reported increased prolactin secretion in lactating rats allowed to see and smell their pups but not suckle them.

The effect of the suckling stimulus on endocrine parameters other than corticoid secretion is evident in changes in ovarian weight. Increased ovarian weight in rats suckling two pups relative to rats suckling six pups corresponds with previous observation of Minaguchi and Meites (1960), who reported lower ovarian weight and pituitary LH content in lactating rats relative to cyclic controls. Rothchild (1960) reported decreased ovarian weight in suckled rats and suggested that reduced ovarian weight during lactation in rats may result from an inhibitory effect on suckling generated stimuli on gonadotrophin release. Parlow (1964) reported reduced pituitary and blood serum concentration of FSH and LH in castrate lactating rats relative to castrate postpartum rats void of litters. Clapp (1937) and Wiltbank and Cook (1958) reported a longer interval between calving and first estrus in suckled cows relative to milked ones.

Adrenal ascorbic acid and cholesterol depletion have also been utilized as indices of ACTH release in response to suckling. We did not demonstrate changes in average adrenal ascorbic acid concentration due to acute or chronic suckling. Jones *et al.* (1953) reported decreased

adrenal cholesterol concentration but not adrenal ascorbic acid in pregnant rats relative to nonpregnant controls. However, Anderson and Turner (1962) reported a reduction in adrenal ascorbic acid concentration during pregnancy and the first six days of lactation relative to nonpregnant controls. After the sixth day of lactation adrenal ascorbic acid returned to levels characteristic of non-pregnant rats. Tabachnick and Trentin (1951) reported significant reduction in adrenal ascorbic acid concentration in suckled mice relative to nonsuckled controls. Knobil and Briggs (1957) also observed decreased adrenal ascorbic acid and cholesterol concentration during pregnancy in rats.

SUMMARY AND CONCLUSION

Lactating rats remained with their litters until day seven of lactation when they were subjected to: (a) continued suckling, (b) 12.5 hours nonsuckling, or (c) 12.0 hours of nonsuckling followed by 0.5 hour of suckling. Plasma corticosterone concentration of rats continually suckling two or six pups was 23.8 and 29.3 µg% respectively. Following 12.5 hours of nonsuckling plasma corticosterone was reduced to 19.7 and 20.2 µg% for rats suckling two and six pups respectively. Corticosterone released in response to suckling appeared to be related to intensity of the suckling stimulus, since 0.5 hour of suckling following 12.0 hours of nonsuckling increased plasma corticosterone one and three-fold in rats suckling two and six-pup litters respectively. Adrenal ascorbic acid concentration was unaffected by the suckling regime. Rats suckling six-pup litters had greater mammary weight than comparable average of rats suckling two pups. In both two and six-pup groups, 12.5 hours of nonsuckling resulted in accumulation of milk and increased mammary weight. Following 12.0 hours of nonsuckling, 0.5 hour of suckling was inadequate to significantly decrease mammary weight. Average ovarian weight of rats suckling

two pups was significantly greater than comparable average for rats suckling six-pup litters.

Plasma corticosterone concentration of rats killed on day 11 of pregnancy averaged 28.3 µg% significantly lower ($p < 0.05$) than comparable values at day 19 of pregnancy (40.3 µg%) or at parturition (40.9 µg%). Plasma corticosterone concentration in rats killed on days two or 15 of lactation was not different ($p > 0.05$) from comparable values on day 19 of pregnancy or at parturition. Adrenal corticosterone increased from 4.3 µg/100 mg in rats killed on day 11 of pregnancy to 5.6 µg/100 mg on day 19 of pregnancy, and remained unchanged until day 15 of lactation when it increased to 7.6 µg/100 mg. Failure of plasma corticosterone concentration to increase at parturition relative to day 19 of pregnancy, suggests that the adrenal-pituitary activity reaches a peak well in advance of pregnancy. Mammary growth during pregnancy coincided with increased corticosterone concentration.

Prior to milking, serum corticoid concentration in Holstein cows averaged 3.9 ng/ml and was increased to 8.1 and 11.5 ng/ml at five and 15 minutes after the start of milking respectively. By 60 minutes postmilking serum corticoid declined to premilked values. Serum corticoid concentration of nonmilked cows, sampled while their pairmates were milked, increased from 4.6 ng/ml at ten minutes before milking to 6.7 and 8.2 ng/ml at five and

one minute before milking, respectively, remained high through 15 minutes postmilking and declined thereafter. Serum corticoid concentration of nonmilked cows remained unchanged on day one, but was increased by five minutes before milking on day two. The results suggest that milking per se and exteroceptive stimuli are capable of releasing corticoids in cows.

In Holstein heifers, serum corticoid concentration averaged 5.0 ng/ml from 26 to one day prepartum, increased to 10.3 and 16.7 ng/ml 12 hours before and at parturition respectively. Thereafter serum corticoid concentration decreased to 5.1 ng/ml approximately 12 hours postpartum. Although serum corticoid showed marked variation during the first nine days postpartum the average concentration of 5.6 ng/ml was not different from 5.0 ng/ml for the period 26 to one day prepartum. Increased serum corticoid concentration immediately before or at parturition is consistent with its role in the initiation of parturition and lactation. Increased corticoid concentration may result from stimuli associated with labor or decreased catabolism of corticoid during the latter stages of pregnancy.

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APPENDICES

APPENDIX 1

SPECIAL WASH PROCEDURE FOR GLASSWARE USED IN FLUOROMETRIC DETERMINATION OF RAT PLASMA CORTICOSTERONE

Clean dishpan with alconox (Scientific Prod., Evanston, Ill.).

Wash glassware in hot water with alconox four times.

Rinse in tap water (cool) eight times.

Rinse in distilled water five times.

Rinse in glass double distilled water four times.

Rinse with acetone two times.

Allow to dry.

APPENDIX 2

FLUORESCENT REAGENT FOR CORTICOSTERONE DETERMINATION

Special wash thick-walled glass stoppered bottle.

Add sulfuric acid : ethanol (3:1) mixture.

Cool mixture in ice bath and allow to stand in the dark at least 12 hours before use. Keep stored in dark during use.

Sulfuric acid 95.0 - 98.0%; 1.84 specific gravity (Mallinckrodt Chemical Works, St. Louis, Mo.).

Ethanol--200 proof--"Gold Shield Alcohol" (Commercial Solvents Corp., Terre Haute, Indiana).

APPENDIX 3

COMPOSITION OF LIQUID SCINTILLATION FLUIDS

A. Steroid scintillation fluid.

7.5 g PPO (Packard Instrument Co., Inc., Downers Grove, Ill.).

0.075 g POPOP (Packard Instrument Co., Inc., Downers Grove, Ill.).

120 g Napthalene (Mallinckrodt Chemical Works, St. Louis, Mo.).

500 ml zylene (Mallinckrodt Chemical Works, St. Louis, Mo.).

500 ml p-dioxane (Eastman Kodak Co., Rochester, N.Y.).

Mix until dissolved.

B. Bray's solution.

4 g PPO.

0.2 g Dimethyl POPOP (Packard Instrument Co., Inc.).

60 g Napthalene.

20 ml Ethylene glycol (Mallinckrodt Chemical Works).

100 ml Methanol (Mallinckrodt Chemical Works).

816 ml p-dioxane.

Mix until dissolved.

APPENDIX 4

PREPARATION OF DEXTRAN-COATED CHARCOAL FOR DETERMINING SERUM CORTICOID

1.25 g Charcoal--Carbon Decolorizing Neutral Norit (Fisher Scientific Co., Fair Lawn, N.J.).

0.125 g Dextran 150 (Pharmacia Fine Chemicals, Uppsala, Sweden).

Dissolved in 100 ml double glass distilled water.

APPENDIX 5

INDOPHENOL-ACETATE SOLUTION FOR DETERMINING ADRENAL ASCORBIC ACID

A. 2,6-dichlorophenol indophenol solution.

Dissolve 20.0 mg 2,6-dichlorophenol indophenol in 500 ml glass double distilled water at 85-95°C.

Store in cold and prepare fresh every two weeks.

B. Sodium acetate solution.

Dissolve 22.65 g sodium acetate $3H_2O$ (A. R. grade) in 500 ml double glass distilled water. Adjust pH to 7.0 ± 0.2 with 6% acetic acid.

Store in cold and prepare fresh each week.

C. Indophenol-acetate solution.

Mixture of 2,6-dichlorophenol indophenol solution : sodium acetate solution (1:1) mixture.

Store in cold and prepare fresh every three days.