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ABSTRACT

CORTICOID BINDING IN BOVINE MAMMARY
TISSUE SLICES AND SERA

By

Ronald C. Gorewit

Various unlabelled corticoids significantly reduced binding of tritiated cortisol and dexamethasone to mammary tissue slices from lactating cows separated into 700 x g supernatant and precipitate fractions. Similarly, unlabelled cortisol and dexamethasone significantly reduced tritiated cortisol and dexamethasone binding to components within 700 x g fractions of mammary tissue slices from virgin heifers, 1-month prepartum and dry (nonpregnant, nonlactating) cows. Unlabelled progesterone, testosterone and 17β-estradiol had no effect on tritiated corticoid binding in mammary tissue slices.

Mammary tissue slices from lactating cows, virgin heifers, 1-month prepartum and dry cows, incubated at 37°C with various concentrations of tritiated cortisol and dexamethasone bound these hormones with high affinity ($Kd \sim x$ $10^{-10}M$). There were 1263 and 1855 molecules of cortisol and dexamethasone bound per mammary cell, respectively, in mammary tissue slices from lactating cows; whereas, virgin

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heifers bound 413 and 651 molecules of cortisol and dexa-
methasone per mammary cell, respectively; dry cows bound
536 and 336 molecules of cortisol and dexamethasone per
mammary cell, respectively; and one-month prepartum cows
bound 542 molecules of cortisol per mammary cell. All
cattle bound approximately 2.0 times as many molecules of
dexamethasone as cortisol in mammary tissue slices.

Mammary tissue slices from cattle in all physiological
states examined contained a major nonspecific component
which bound cortisol in both 700 x g tissue fractions.
Nonspecific binding could not be completely reduced by
repeated washing of tissues, biochemical fractionation
procedures or treatment of tissue fractions with florisil
or dextran coated charcoal.

Incubation of mammary tissue slices from lactating cows
at 4°C reduced the total number of molecules of cortisol
bound to high affinity binding sites by 82% compared with
similar measurements made at 37°C. However, the dissocia-
tion constant for the high affinity binding component was
not significantly changed by lowering the temperature.

Thin-layer chromatography of tritiated cortisol bound
in 700 x g supernatant, indicated that the majority of
radioactivity was authentic cortisol. Macromolecules which
specifically bound cortisol in 700, 15,000 and 100,000 x g

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Ronald C. Gorewit

supernatants and 700 x g precipitate tissue fractions of tissue slices from lactating cows were isolated by gel filtration chromatography. Enzyme digestion experiments and treatment with parachloromercuribenzoate indicated that the macromolecules binding cortisol were protein(s) containing sulfhydryl groups. Uptake of cortisol by these protein(s) was not inhibited by 10^{-5} mM Ouabain, which suggested that transport of cortisol into mammary tissue was not linked to Na^+/K^+ active transport.

Approximate molecular weight determinations suggested that the corticoid binding protein(s) of mammary tissue had an approximate molecular weight in the range of 2.5 x 10^5 to 3 x 10^6. In contrast, the binding protein for corticoids in blood had an approximate molecular weight in the range of 6 to 8 x 10^4. Thus, the proteins binding cortisol in mammary tissue and blood sera were dissimilar. Further evidence for the unique character of the mammary cortisol binding protein(s) was indirectly provided by experiments designed to compare the amount of binding of tritiated steroids to bovine sera and mammary tissue slices. Bovine sera bound approximately five times more cortisol and progesterone per mg protein than dexamethasone. In contrast, mammary tissue slices bound more total molecules of dexamethasone than cortisol.

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Ronald C. Gorewit

Cell-free preparations of 700 x g supernatant and precipitate fractions specifically bound cortisol. Although cell-free preparations of 100,000 x g precipitates bound large amounts of ^{3}H-cortisol, this binding was not specific because addition of unlabelled cortisol or dexamethasone consistently failed to reduce binding of ^{3}H-cortisol.

Unlabelled cortexelone, cortisol, triamcinolone and dexamethasone inhibited ^{14}C-glucose incorporation into mammary tissue slices from lactating cows. As the concentration of corticoids increased, inhibition of ^{14}C-glucose uptake increased in a dose response relationship. Correlation analyses showed that corticoid binding was related to the ability of corticoids to inhibit ^{14}C-glucose uptake in tissue slices. When unlabelled cortisol and cortexelone were simultaneously added to mammary tissue slices from lactating cows glucose uptake was reduced by only 4% when compared with nonhormone treated controls. Cortexelone interacted with cortisol to increase ^{14}C-glucose uptake. These results further suggested that specific corticoid binding to lactating mammary tissue was related to the physiological action of corticoids on glucose uptake.

The external pudic artery-mammary vein concentration differences for corticoids were 5.81 ng/ml and 2.89 ng/ml at 6 and 12 minutes after the start of milking. These

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intervals corresponded to the times when serum cortisol
concentrations were maximal after the milking stimulus, and
provided further indirect evidence that cortisol was associ-
ated with a lactational event.

In summary, evidence presented in this dissertation
suggested that mammary tissue slices possessed receptor
molecules which were capable of specifically binding corti-
coids. The fact that tissue slices from lactating cows
bound more molecules of corticoids than tissue from cattle
in other physiological states and that binding and uptake
of corticoids appeared related to events associated with
milk secretion, may form the basis for further studies to
determine the specific role which corticoids play in lacta-
tion.

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CORTICOID BINDING IN BOVINE MAMMARY

TISSUE SLICES AND SERA

By

Ronald C. Gorewit

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Physiology

1975

To My Parents

and

Beloved Sister

ACKNOWLEDGEMENTS

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INTRODUCTION

Hormones of the adrenal cortex stimulate mammary
development and are associated with the initiation and
maintenance of lactation. The mechanism by which corticoids
exert their physiological action is obscure, but it is be-
lieved that the initial event requires binding of hormone to
receptor molecule(s) within the mammary cell with eventual
action on deoxyribonucleic acid (DNA).

Corticoid hormone receptors have been described for a
number of mammalian tissues including thymus, liver, brain
and blood. They have only recently been found in mammary
tissue and cultured mammary cells. For example, lactating
rat, mouse, and vole mammary tissues were capable of binding
either radioactive cortisol or radioactive synthetic corti-
coids. Rat mammary tumors and bovine mammary cells cul-
tured in vitro also bound radioactive corticoids. This
binding was considered specific because it was reduced by
unlabelled corticoids and binding site(s) were saturated at
physiological hormone concentrations.

The primary objective of the research described in this
dissertation was to determine whether corticoid hormones
would specifically bind to fresh mammary tissue slices from

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lactating cattle. Once this was established, attempts were made to demonstrate specific corticoid binding and uptake in animals in other physiological states. Further attempts were made to compare some physicochemical characteristics of corticoid receptor molecules in lactating mammary tissue slices with corticoid receptors in blood serum. It is hoped that this work will help resolve certain unanswered questions regarding the mechanism of corticoid action on mammary tissue.

REVIEW OF LITERATURE

Mammary Development

It is generally recognized that hormones of the adrenal cortex stimulate mammary development secondarily through synergism with pituitary, ovarian, thyroid and pancreatic hormones. Evidence for this concept is given below.

In Vivo Experiments

In vivo experiments designed to determine the effects of hormones on mammary development and growth have been carried out by one of two methods. The first method involved measurement of mammary growth after exogenous administration of hormones to animals with intact endocrine glands. The other experimental method involved observing changes in mammary gland development and growth after removal of various endocrine glands, with or without hormone replacement therapy.

Kumareson et al. (1967) and Griffith and Turner (1963) examined the effects of exogenously administered corticoids on mammary growth in intact animals. Corticosterone given throughout pregnancy in rats stimulated mammary cell numbers (DNA content) 23% and metabolic activity (ribonucleic acid,

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RNA) 52% at the 19th day of pregnancy. Mammary growth
equivalent to that observed during lactation was accom-
plished in intact rats with estradiol benzoate and progester-
one treatment for 19 days followed by injection of growth
hormone and cortisol.

Anderson and Turner (1962a) demonstrated the synergis-
tic effects of corticoids and ovarian hormones on mammary
growth of adrenalectomized-ovariectomized rats. Predniso-
lone and cortisol enhanced mammary growth in operated rats
when administered simultaneously with estrogen and progester-
one. These corticoids given alone did not enhance mammary
growth in adrenalectomized-ovariectomized rats previously
treated with ovarian steroids. These results were substan-
tiated further by Hahn (1967) who reported that corticoster-
one synergized with estradiol and progesterone to promote
mammary growth in ovariectomized rats.

Lyons et al. (1958) and Nandi (1958) demonstrated that
corticoids synergized with ovarian and pituitary hormones
to enhance mammary duct growth and lobule-alveolar develop-
ment. This work was carried out on hypophysectomized-
ovariectomized-adrenalectomized (triply-operated) rats and
mice. In these studies, replacement therapy with estrogens,
growth hormone and deoxycorticosterone or prednisolone ace-
tate led to full duct growth. However, neither estrogen,
deoxycorticosterone acetate nor prednisolone acetate

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stimulated ductual growth when administered alone. Growth hormone plus estrogen stimulated ductual growth, but corticoid was required before ductual growth typical of prepuberal and puberal ages was duplicated. Treatments with estrogen, progesterone, prolactin, growth hormone and corticoid resulted in full lobule-alveolar development. In contrast to these results, Hahn and Turner (1967) showed that the depression of mammary growth in triply-operated rats was not significantly greater than the depression in mammary growth observed for hypophysectomized rats. Further experimental evidence for the synergism between corticoids, ovarian and pituitary hormones on stimulating mammary development was provided by Cowie et al. (1966a). These workers demonstrated lobule-alveolar development, comparable to mid-pregnancy in hypophysectomized-ovariectomized goats after treatments with hexestrol, progesterone, growth hormone, prolactin, and adrenocorticotropic hormone (ACTH).

Work in other laboratories has shown the synergism between corticoids, thyroid and pancreatic hormones on enhancing mammary development. For example, Hahn (1967) showed that corticosterone, thyroxine, bovine growth hormone and ovine prolactin stimulated mammary growth in hypophysectomized rats to a level 97% above control animals. Moreover, Ahren and Jacobsohn (1957) presented histological evidence that glucocorticoids stimulated mammary growth when

administered with estrogen, progesterone and insulin in hypophysectomized rats.

In Vitro Experiments

In general, experiments carried out on mammary tissue explants from mice and rats, cultured in vitro in medium containing hormone supplements, have confirmed the results of in vivo work on hormonal requirements for normal mammary development and growth. Ovarian, pituitary, thyroid, pancreatic and adrenal hormones were necessary for normal development of mammary explants (Lasfargues and Murray, 1959; Prop, 1966; Bern and Rivera, 1960; Rivera, 1963, 1964a, b,c; Rivera and Kahn, 1970; Ranadive and Chapekar, 1964; Ichionose and Nandi, 1964, 1966; Gadkari et al., 1968; Dilley and Nandi, 1968; El Darwish and Rivera, 1970; Dilley, 1971 and Forsyth, 1971).

Initiation of Lactation

In Vivo Experiments

Serum glucocorticoids in many species remain unchanged or are slightly reduced during most of pregnancy. However, serum concentrations of corticoids increased along with growth hormone, prolactin and estrogens, while progesterone decreased shortly before parturition in cows (Adams and Wagner, 1970; Heitzman et al., 1970; Robinson et al., 1970;

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Ingalls et al., 1973 and Smith et al., 1973), guinea pigs
(Gala and Westphal, 1967 and Rosenthal et al., 1969), dogs
(Seal and Doe, 1963), rats (Milkovic and Milkovic, 1963;
Kamoun et al., 1965; Gala and Westphal, 1965a,b and Voogt
et al., 1969), mice (Gala and Westphal, 1967), rabbits
(Gala and Westphal, 1967) and women (Stewart et al., 1961;
Friedman and Beard, 1966; Scholz and Huther, 1971). It is
believed that increased serum levels of growth hormone,
prolactin, estrogens and adrenal cortical hormones and
decreased progesterone levels, just before parturition,
may provide the stimulus for the initiation of lactogenesis
(Cowie and Tindal, 1971). This hypothesis does not apply
for corticoids in ewes and monkies, since elevated secre-
tion of corticosteroids has not been observed before par-
turition in these species (Patterson and Harrison, 1967,
1968; Basset and Thorburn, 1969 and Wolf and Bowman, 1966).

Several workers have succeeded in initiating lactation
in intact pregnant animals with injections of either pro-
lactin or cortisol (Talwalker et al., 1961, rats; Nandi and
Bern, 1961, mice; Talwalker et al., 1961, Meites et al.,
1963 and Friesen, 1966, rabbits; Delouis and Denamur, 1967,
ewes; Tucker and Meites, 1965, cows). Furthermore, Chadwick
and Folley (1962) and Chadwick (1971) increased the sensi-
tivity of mammary glands by treatment with ACTH and adrenal
corticosteroids.

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Lyons (1958) successfully initiated lactation in triply-operated virgin rats. Animals were treated initially with ovarian hormones, prolactin and cortisol to maximize lobule-alveolar development. Following this, milk secretion was initiated in 6 days with a combination of prolactin, deoxycorticosterone acetate or prednisolone acetate. Similarly, Davis and Liu (1969) and Meunier (1962) have shown that cortisol, corticosterone or cortisone restored normal milk secretion in adrenalectomized or adrenalectomized-ovariectomized rats. Ben-David and Sulman (1970) provided further evidence that initiation of lactation was dependent upon prolactin and glucocorticoids in rats. Lactation was induced in normal intact rats with the tranquilizer per-phenazine. However, this response did not occur in adrenal-ectomized animals. Since perphenazine elevated prolactin in blood, the failure of lactogenesis was attributed to a lack of glucocorticoid. In contrast, work by Cowie and Watson (1966b) and Denamur (1969) suggested that rabbits may have no absolute requirement for steroids in the initia-tion of lactation. These workers initiated lactation with prolactin alone in adrenalectomized and ovariectomized-adrenalectomized rabbits. Furthermore, Cowie et al. (1969) reinitiated or maintained lactation in hypophysectomized lactating rabbits while Denamur (1969, 1971) initiated lactation in hypophysectomized or triply-operated pseudo-pregnant rabbits.

In Vitro Experiments

Hormonal requirements for the in vitro initiation of milk secretion in mice have been examined by several workers. For example, Rivera (1964a,b) found that milk secretion in C3H mice was initiated in vitro by corticosterone, aldosterone or cortisol in combination with insulin + prolactin + growth hormone. Similarly, Juergens et al. (1965) reported that a combination of insulin, prolactin and cortisol induced the synthesis of "casein like" phosphoprotein by mid-pregnant C3H mouse mammary explants. Other hormone combinations were ineffective. Maximum casein synthesis occurred after 48 hours of culture. Further support of this observation was provided by Topper (1968) who showed that the maximal rate of induced casein synthesis, which occurred after 48 hours, was approximately 50% of that seen in normal mammary tissues for 10 day postpartum mice.

Corticoid molecular structural requirements for the initiation of lactation in mice have been investigated by Turkington et al. (1967). They compared histological and biosynthetic responses to insulin and prolactin with and without corticoids. The five most effective steroids which supported in vitro milk secretion were 21-deoxycortisol, aldosterone, cortisone, cortisol and prednisolone.

Biochemical evidence has indicated that prolactin, insulin, progesterone and corticoids play roles in the

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synthesis of milk lactose. For example, lactose synthesis is controlled by lactose synthetase, an enzyme which is composed of non-specific galactosyl transferase (A protein) and a specific whey protein (B protein, α-lactalbumin) (Brodbeck and Ebner, 1966). In mice, mammary explant studies by Turkinton and Hill (1969) have indicated that prolactin, insulin and cortisol in combination, induced α-lactalbumin synthesis, but that progesterone selectively inhibited the response. It is believed that the fall in circulating progesterone, which has been observed at the end of pregnancy in a number of mammals, permits stimulation of α-lactalbumin synthesis.

Maintenance of Lactation

In Vivo Experiments

Several investigators have shown that corticoids are rate limiting to lactation in rats. For instance, Thatcher and Tucker (1968) showed that rat pituitary ACTH decreased 68% between days 20 and 36 of lactation. Additionally, Holzbauer (1957) showed that adrenal corticosterone content, which has been used as an index of secretory activity, decreased linearly between days 16 and 32 of lactation in rats. Furthermore, Hahn and Turner (1966), Johnson and Meites (1958) and Talwalker et al. (1960) stimulated lactational performance by 12-27% in intact rats by injections

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of either corticosterone, cortisone acetate or cortisol acetate. In addition to this work, Thatcher and Tucker (1970a,b) markedly reduced the decline in lactation seen in rats by treatment with either cortisol acetate or 9 α-fluoro-predinisolone acetate. Emery et al. (1971) maintained mammary cell nucleic acid content for up to 32 days when intact rats were given glucocorticoids alone or in combination with high fat diets. In contrast to the work described above, Hahn and Turner (1966) and Kowalewski (1969) inhibited lactational performance in early lactation with high doses of corticoids.

Cowie and Tindal (1958) and Anderson and Turner (1962b, 1963a,b,c) examined the effects of adrenalectomy on the lactational performance of goats, rats and mice. These workers found that adrenalectomy impaired milk secretion. Furthermore, Anderson and Turner (1962b, 1963a,b,c) demonstrated that glucocorticoids or mineralocorticoid partially restored milk secretion in adrenalectomized animals. A combination of the two steroids was more effective than either given separately. This suggested that the decline in milk secretion in adrenalectomized rats was in part a result of metabolic defects, which were corrected for by exogenous mineralocorticoid.

Corticoids seem to be rate limiting to lactation in rats as described above; however, most experimental evidence to date suggests that this is not the case for cows.

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For instance, Koprowski and Tucker (1973) observed that cows did not lose their ability to discharge corticoids into serum at milking as lactation progressed. Also, basal concentrations of corticoids in blood remained unchanged throughout lactation. Moreover, Head et al. (1972) exogenously administered flumethazone (6α, 9α-difluoro-16α-methyl prednisolone) to lactating cows beginning at the 28th day of lactation. The corticoid failed to affect daily milk production over a 305 day lactation. The pituitary seemed unresponsive to exogenously administered corticoid during the first 28 days of lactation, since flumethazone failed to depress endogenous plasma corticoids during this interval.

Even though corticoids may not be rate limiting to lactation in cows, work by Patterson and Linzell (1971, 1974) suggested that corticoids were utilized by goats and cows during milk secretion. Mammary glands of preparturient and lactating goats and cows removed an average of 1.1 to 1.3 μg of cortisol per minute from the blood. Metabolic clearance rates of corticoids did not change appreciably; however, corticoid secretion rates were approximately four times greater in lactating goats and cows as compared with pregnant animals. The mammary glands of goats and cows removed approximately 3 to 4% of the secreted cortisol and 2/3 of the unbound hormone which entered the gland.

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In Vitro Experiments

In vitro experiments suggesting the importance of corti-
coids on the maintenance of lactation have been reviewed by
Forsyth (1971). In summary, mammary explants which are pro-
ducing secretory products in response to insulin, corticol
and prolactin fail to do so when one or more of these hor-
mones is omitted from the culture medium.

Binding of Corticoids to Mammary
Tissue and Cells

It is currently believed that, like most steroid hor-
mones, corticoids must bind to receptor molecules within
their respective target tissues before their physiological
action can be exerted. Corticoid binding and corticoid
receptor molecules have recently been demonstrated in mam-
mary tissue and cells.

Emery (1969) and Steyert and Emery (1971) demonstrated
tritiated corticosterone binding and receptor molecule(s) in
rat and cow mammary gland homogenates. Corticosterone was
bound to extranuclear fractions of homogenates which con-
tained mammary cytosol along with microsomes and mitochon-
dria. Fractionation of homogenates by differential
contrifugation and chromatography revealed that tritiated
corticosterone was bound to a protein(s). In a series of
preliminary experiments, Emery (1969) found that the amount

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of radioactive corticosterone bound to rat mammary homogen-
ates increased sixfold at 16 days of lactation when compared
with rats which were in their 13th day of pregnancy. He
also found that mammary homogenate isolated from one lactat-
ing cow bound more molecules of corticoid than the mammary
homogenate of a pregnant cow.

Prevention of suckling in rats lowered the total amount
of corticosterone bound per mg of protein. Since addition
of milk to rat and cow mammary homogenates had little effect
on binding activity, it was concluded that milk precursors
in the cell interfered with binding activity or increased
the lability of the corticosterone binding protein.

Shyamala (1973a) reported specific corticoid binding in
cytoplasmic fractions of lactating mouse mammary tissue.
Unlabelled triamicinolone, fluorocortisol, corticosterone,
cortisol and aldosterone competed with tritiated dexametha-
sone for binding sites. Unlabelled 17-β estradiol, testo-
sterone, androstendione and spironolactone were without
effect. This suggested that binding was specific for corti-
coids. Proteolytic enzymes and mercurials reduced or in-
hibited binding, but DNAse and RNAse were without effect,
whicn suggested that the receptor molecule(s) was a protein.
Receptor molecule(s) sedimented near 6S in low ionic
strength sucrose and at 4S in high ionic strength sucrose.
The Kd for the dexamethasone-receptor complex was 6 x 10^{-9} M

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at 0-4°C. Scatchard plots of binding data revealed a single straight line with high slope, which suggested a single class of specific receptors for corticoid. Shyamala (1973b) also found glucocorticoid receptors in cytosol fractions of mouse mammary tumor tissue. The binding protein(s) had high affinity for corticoid ($Kd = 4 \times 10^{-9}$ M, dexamethasone) and displayed a limited number of binding sites. The binding sites saturated at hormone concentrations of 1×10^{-8} M.

Gardner and Witliff (1973a,b) and Turnell et al. (1974b) reported the presence of specific molecules which bound tritiated triamcinolone acetonide in cytosol fractions from normal lactating rat and vole mammary glands and rat mammary tumors. The receptors were protein(s), exhibited sedimentation coefficients of 7 to 8S and dissociated into lower molecular weight components which sedimented at 4 to 5S when separated on sucrose gradients containing 0.4M KCl. Competitive hormone binding experiments showed specificity toward glucocorticoids. However, progesterone and aldosterone reduced tritiated triamcinolone binding. The Kd for the hormone-receptor complex was in the range of 10^{-7} to 10^{-9} M. Parachloromercuribenzoate reduced binding, which suggested that sulfhydryl groups played a role in the binding reaction. Moreover, mammary cytosol fractions from virgin and pregnant rats contained fewer binding sites when compared with the number found in lactating rats.

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Tucker et al. (1971) demonstrated cortisol binding receptors in bovine mammary cells cultured in vitro. In this work, 77% of the amount of tritiated cortisol taken up by the cells was present in the cytosol after incubation at 2 or 37°C. The binding of tritiated cortisol was reduced by unlabelled cortisol, corticosterone, deoxycorticosterone, aldosterone and dexamethasone. Progesterone and 17 α-hydroxyprogesterone also reduced tritiated cortisol binding. In contrast, estradiol-17-β and testosterone did not affect binding. Scatchard plots revealed that mammary cell cytosol fractions contained three components which bound cortisol. One of the components had almost zero slope and was practically unsaturable. This component was thought to represent nonspecifically bound hormone. The other two binding components had high affinity for cortisol. For example, the major high affinity component had a dissociation constant (Kd) for cortisol equal to 5×10^{-8} M. This component bound approximately 5,400 molecules of cortisol per cell. The third component had a Kd = 2×10^{-9} M for cortisol, but it bound only approximately 500 molecules of cortisol per cell. Lowering the incubation temperature from 37 to 2°C had little affect on dissociation constants, but reduced the number of molecules bound by approximately 33%. Most of the radioactivity in the cytosol was in the form of unmetabolized cortisol; however, 18% of the radioactivity

was associated with metabolite(s). The majority of tri-
tiated cortisol was found in cytoplasmic fractions within
15 minutes of incubating the cells with labelled hormone.
Radioactive cortisol moved toward the nucleus during the
first 60 minutes of incubation at 37°C. Heating the cytosol
to 100°C destroyed the binding of tritiated cortisol in
cytosol fractions.

Tucker et al. (1971) also demonstrated cortisol bind-
ing in nuclear fractions of cultured bovine mammary cells.
Twenty-one percent of the total tritiated cortisol, taken
up by the cells, was associated with nuclear fractions after
incubation at either 37°C or 2°C. Unlabelled dexamethazone,
cortisol, and corticosterone reduced tritiated cortisol
binding to nuclear receptor sites. Like the mammary cyto-
sol, Scatchard plots of the nuclear fractions contained
three components which bound cortisol. One component was
virtually unsaturable and was believed to represent non-
specifically bound hormone. The second component had a
high affinity for cortisol (Kd = 4 x 10^{-8} M). The third
component had a Kd = 1 x 10^{-9} M for cortisol. Heating the
cells to 100°C for 10 minutes destroyed binding to nuclear
fractions. Thirty-one per cent of the cortisol bound to
nuclear fractions was in the form of metabolized hormone.
In addition, most of the radioactive cortisol incorporation
into the nuclei occurred within 15 minutes of incubation.

These workers also observed minimal uptake of tritiated cortisol into microsomal and mitochondrial fractions of cultured mammary cells. The uptake in these fractions accounted for only 0.7 and 9.7%, of the total amount of radioactivity incorporated per cell respectively.

Binding of Corticoids to Thymus, Liver, Brain and Blood

Glucocorticoids play important roles in regulating the physiology and biochemistry of the thymus, liver and brain. For instance, glucocorticoids cause atrophy of the thymus and other lymphoid tissues (Yates, 1974; Ganong, 1969 and Litwack and Singer, 1972), regulate carbohydrate and fat metabolism in the liver (Weber et al. 1966, 1968) and affect carbohydrate metabolism, growth and excitability of brain tissue (Frieden and Lipner, 1971; Ganong, 1969; Axelrod, 1971; Vernadakis and Woodburg, 1971 and DeVellis et al., 1971). Furthermore, corticoids are transported to these target tissues via blood. Corticoids are bound to blood proteins which provide for corticoid transport. The primary transport protein for corticoids in blood is corticoid binding globulin (CBG) or transcortin (Westphal, 1971). Corticoid binding mechanisms have been extensively examined in all the tissues described above (Munck and Brinck-Johnsen, 1968; Litwack and Singer, 1972; Koch et al., 1972; Turnell

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et al., 1974a; Munck and Wira, 1971; Schaumburg, 1970, 1972;
rat thymus), (Snart et al., 1970; Gardner and Tomkins, 1969
and Baxter and Tomkins, 1971a,b; rat liver), (McEwen and
Plapinger, 1970; McEwen et al., 1972 and Grosser et al.,
1973; rat brain), and (Sandberg et al., 1966; Seal and Doe,
1961, 1962, 1963 and 1966 and Muldoon and Westphal, 1967;
human blood). The results of this work are summarized below.

Unlabelled cortisol, corticosterone, deoxycorticosterone
and cortexelone (11-deoxycortisol) were capable of reducing
either radioactive cortisol, corticosterone, dexamethasone
or triamcinolone acetonide binding in thymus, liver and
brain. In addition to these hormones, various unlabelled
fluorinated corticoids such as dexamethasone, dexamethasone-
21-phosphate, 9 α-fluoroprednisolone and triamcinolone
acetonide reduced the binding of radioactive corticoids to
binding sites in thymus, liver and brain. In contrast,
unlabelled fluorinated corticoids did not reduce radioactive
corticoid binding to CBG of blood to the extent seen in
thymus, liver, and brain (Kolanowski and Pizarro, 1969;
Peets et al., 1969; Baxter and Tomkins, 1971a and Sandberg
et al., 1966). Unlabelled estradiol 17β and 17α-hydroxy
progesterone also reduced corticoid binding in liver,
thymus and brain. This reduction in binding, however,
was not to the degree seen for unlabelled corticoids.
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corticoid binding in liver, thymus and brain tissue. Progesterone greatly reduced corticoid binding to CBG at 37°C when compared to experiments carried out at 4°C. Estradiol-17β and testosterone reduced corticoid binding to CBG, but not to the extent seen with either corticoids or progesterone.

Dissociation constants for corticoid-receptor complexes, calculated from Scatchard plots of corticoids bound to thymus, liver and brain were in the range of 10^{-8} to 10^{-10} M. Purified CBG from human blood sera had a Kd for cortisol of 3×10^{-8} M and 2×10^{-9} M at 37° and 4°C, respectively. Thymus, liver and brain bound approximately 2400-5000 total molecules of corticoid per cell. Purified CBG bound one molecule of steroid per molecule of protein.

Several investigators have isolated the glucocorticoid receptors from thymus, liver, brain and blood (Munck and Wira, 1971 and Schaumberg, 1970, 1972, rat thymus; Litwack and Singer, 1972; Beato et al., 1969, 1970a,b, 1971; Gardner and Tomkins, 1969 and Baxter and Tomkins, 1971a,b, rat liver; McEwen and Plapinger, 1970 and McEwen et al., 1972, rat brain; and Seal and Doe, 1961, 1963, 1966 and Muldoon and Westphal, 1967, human blood). In summary, the receptors all appeared to be proteins since they were destroyed by proteolytic enzymes, mercurials and heating at 100°C for 10 minutes. The sedimentation coefficients of the binding

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proteins ranged from 4 to 8S on sucrose gradients and varied according to the ionic strength of the buffers used. The molecular weights of the binding proteins ranged from 50-250,000, again depending upon the particular experimental conditions utilized.

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MATERIALS AND METHODS

In Vitro Experiments

Routine Procedures

Mammary tissue samples weighing approximately 500 mg were randomly removed from eight areas of the mammary glands of Holstein heifers or cows within 15-30 minutes after slaughter. The samples were immediately transferred to either ice cold 0.01M Tris-EDTA buffer (0.01M Trizma base, and 0.01M ethylenediamine tetracetic acid, Sigma Chemical, adjusted to pH 7.4 with 6N HCl) or to a 1:1 mixture of medium 199:Eagle's minimal essential medium (MEM) at pH 7.4. Within 1-2 hours, the tissue samples were further cut into approximately 0.2-0.4 mm^3 pieces and dispersed into tubes containing either 3 ml of Tris-EDTA buffer or medium 199:MEM. Single pieces from each of the original eight tissue samples were placed in each assay tube. Each treatment usually was conducted in quadruplicate on a given animal, and no more than one animal was used per day. Treatments consisted of incubating tissue with 40 µl of 100% ethyl alcohol contain-ing various radioactive and/or unlabelled steroids. Incuba-tion was carried out for 1 hour at 37°C with intermittent

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shaking. Tissues were then washed 5 times with 5 ml of either Tris-EDTA buffer or Medium 199:MEM at 4°C, and homogenized (Polytron homogenizer, Brinkmann Instruments) in three 5 second bursts at 10 second intervals. The homogenate was routinely centrifuged at 700 x g for 10 minutes at 4°C, and the supernatant decanted into scintillation vials containing 10 ml of scintillation fluid (Appendix A). Samples were counted in a Nuclear-Chicago liquid scintillation spectrometer, and external standardization was used to correct for quench (Appendix B). Isotopic steroids were checked for radiochemical purity as previously described by Tucker et al. (1971). The DNA (Deoxyribonucleic acid) content of the ethyl alcohol extracted 700 x g precipitates was quantified according to the procedures in Appendix C, and the data usually were expressed as DPM/µg DNA (disintegrations per minute per microgram DNA).

Hormone Binding

Preliminary Competition Experiments

In preliminary experiments, tissue slices weighing 500 mg were taken from four lactating cows. The tissues were either kept whole, halved, quartered or scissor minced and incubated for 1 hour at 37°C in Medium 199:MEM containing either 2.7×10^{-9} M ^{3}H-cortisol (1,2 ^{3}H-cortisol, 44 ci/mmole,

New England Nuclear) plus ethyl alcohol or 2.7×10^{-9} M ^{3}H-cortisol plus 6.7×10^{-8} M unlabelled cortisol (Sigma Chemical). After incubation, tissues were homogenized, centrifuged at 700 x g and radioactivity determined as described above.

Specificity of Corticoid Binding in Mammary Tissue Slices from Lactating Cows

1) Competition Experiments

Specificity of ^{3}H-cortisol binding in mammary tissue slices from four lactating cows was determined by adding 6.7×10^{-8} M of various unlabelled steroids simultaneously with 2.7×10^{-9} M ^{3}H-cortisol. The unlabelled hormones were cortisol, dexamethasone, progesterone, 17-β estradiol and testosterone (Sigma Chemical). Control tubes received ethyl alcohol plus 2.7×10^{-9} M ^{3}H-cortisol. After 1 hour incubation at 37°C, the 700 x g supernatant and precipitate fractions were isolated and counted for radioactivity. A similar experiment was conducted in four additional lactating cows except that 2.7×10^{-9} M 1, 2, 4-^{3}H-dexamethasone (Schwartz/Mann; 5.2 ci/mmole) was used in place of ^{3}H-cortisol.

2) Scatchard Analysis

Results of the above experiments showed binding of ^{3}H-cortisol and ^{3}H-dexamethasone was decreased by unlabelled

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cortisol and dexamethasone. In order to determine further characteristics of this inhibition, dissociation constants (Kd's) and number of binding sites were determined in mammary slices from four lactating cows incubated at 37°C or 4°C with 0.005 to 4.0 x 10^{-8} M of a combination of ^{3}H-cortisol and unlabelled cortisol. Other mammary slices were collected from four additional cows and incubated at 37°C with 0.005 to 4 x 10^{-8} M of a combination of 1, 2-^{3}H-dexamethasone (Amersham/Searle; 27 ci/mmole) and unlabelled dexamethasone. The amount of cortisol and dexamethasone bound in the 700 x g supernatant and precipitates was analyzed from Scatchard plots (Scatchard, 1949). Scatchard plots were corrected for nonspecific binding by the transformation given in Appendix D.

Corticoid Binding in Mammary Tissue Slices from Virgin Heifers, 1-Month Prepartum, Lactating and Dry Cows (nonpregnant, nonlactating)

1) Competition Experiments

Virgin Heifers

Specificity of ^{3}H-cortisol binding in mammary tissue slices from four virgin heifers was determined by adding 6.7 x 10^{-8} M of various unlabelled steroids simultaneously with 2.7 x 10^{-9} M ^{3}H-cortisol. The unlabelled hormones were cortisol, dexamethasone, progesterone, 17-β estradiol and testosterone (Sigma Chemical). Control tubes received ethyl

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alcohol plus 2.7 x 10^{-9} M ^{3}H-cortisol. After 1 hour incubation at 37°C, the 700 x g supernatant and precipitate fractions were isolated and counted for radioactivity as described previously. Additional experiments were conducted similarly on the same heifers, except that 2.7 x 10^{-9} M 1, 2, 4-^{3}H-dexamethasone (Schwartz/Mann; 5.2 ci/mmole) was used in place of ^{3}H-cortisol.

1-Month Prepartum, Lactating and Dry Cows

Experiments similar to those described above were carried out using six, 1-month prepartum cows; five, lactating cows; and four, dry cows (nonpregnant, nonlactating).

2) Scatchard Analysis

In order to quantify corticoid binding in mammary tissue from cattle of various physiological states, dissociation constants (Kd's) and number of binding sites were determined for cortisol and dexamethasone in virgin heifers, 1-month prepartum, lactating and dry cows.

Virgin Heifers

Mammary slices from four virgin heifers were incubated at 37°C with 0.005 to 4.0 x 10^{-8} M of a combination of ^{3}H-cortisol and unlabelled cortisol. Other mammary slices were collected from these heifers and incubated at 37°C with 0.005 to 4.0 x 10^{-8} M of a combination of 1, 2-^{3}H-dexamethasone (Amersham/Searle; 27 ci/mmole) and unlabelled

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the 700 x g supernatant and precipitates was analyzed from
Scatchard plots (Scatchard, 1949). Scatchard plots were
corrected for nonspecific binding according to the pro-
cedures given in Appendix D.

1-Month Prepartum, Lactating and Dry Cows

Experiments similar to those described above were
carried out using four, 1-month prepartum cows; four,
lactating cows; and 4, dry cows, except that Scatchard analy-
sis was omitted for ^{3}H-dexamethasone binding in 1-month
prepartum cows.

Metabolism of ^{3}H-cortisol

Mammary tissue slices from four lactating cows were
incubated with 1.3 x 10^{-7} M ^{3}H-cortisol and after washing
and homogenization, 0.6 ml of the 700 x g supernatants plus
0.4 ml of 40% sucrose were applied to Sephadex G-200 columns
and eluted with Tris-EDTA buffer pH 7.4. Those fractions
containing the ^{3}H-cortisol-receptor complex and those con-
taining free ^{3}H-cortisol were extracted with methylene
chloride. This removed over 90% of the radioactivity in
both fractions. Extracts were dried under N_2, dissolved in
ethyl alcohol and 50 µl samples were placed on silica-gel
thin-layer chromatograms (Eastman 6060 plates) and developed
as previously described by Tucker et al. (1971).

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Physicochemical Characterization of
Corticoid Receptors in Tissue
From Lactating Cows

Gel Filtration Chromatography

1) Mammary Cytosol Fractions

700 x g supernatant

Fresh mammary tissue slices from four lactating cows
were incubated for 1 hour at 37°C with either 2.7×10^{-9} M
of ^{3}H-cortisol or 2.7×10^{-9} M ^{3}H-cortisol plus 6.7×10^{-8} M
unlabelled cortisol. After routine washing, homogenization,
and centrifugation, a 0.6 ml sample of the 700 x g super-
natant was added to 0.4 ml of 40% sucrose solution and the
mixture was applied to 1.5 x 20 cm columns of Sephadex G-25
(fine, A.B. Pharmacia). The columns were eluted with Tris-
EDTA buffer pH 7.4. Column operations were carried out at
4°C, and 3 ml fractions were collected. One ml aliquots
were counted for radioactivity, and the remaining aliquots
were assayed for protein by the method of Lowry et al. (1951),
modified by Oyama and Eagle (1956) (Appendix E).

15,000 and 100,000 x g supernatants

To compare ^{3}H-cortisol binding in 700, 15,000 and
100,000 x g supernatant fractions the following was done.
Mammary slices from four lactating cows were incubated, as
above, with 1.3×10^{-7} M ^{3}H-cortisol, washed, homogenized
and centrifuged at 700 x g for 30 minutes. A 0.6 ml sample

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1) Enzymes

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of the 700 x g supernatant was applied to and eluted from G-200 Sephadex columns as described above. The remaining portion of the supernatant was centrifuged at 15,000 x g for 30 minutes and a sample of this supernatant was chromatographed on Sephadex G-200. The remaining supernatant fraction was centrifuged at 100,000 x g for 1 hour. A 0.6 ml sample of this supernatant was chromatographed.

2) <u>Mammary Nuclear Fractions</u>

<u>700 x g precipitate</u>

To determine if binding of ^{3}H-cortisol occurred in protein fractions of 700 x g mammary tissue precipitates (nuclei), the following experiment was conducted. Mammary slices from four lactating cows were incubated with 1.3 x 10^{-7} M ^{3}H-cortisol plus ethyl alcohol or ^{3}H-cortisol plus unlabelled cortisol (2.4 x 10^{-6} M). After washing, homogenization and centrifugation at 700 x g for 30 minutes, the pellet was extracted with 2 ml of 0.3 M KCl for 1 hour at 37°C. The extract was centrifuged at 50,000 x g for 20 minutes, 1 ml of the supernatant was layered on a Sephadex G-25 column and eluted with Tris-EDTA buffer pH 7.4.

<u>Potential Binding Inhibitors</u>

1) <u>Enzymes</u>

Tissue slices from four lactating cows were incubated with ^{3}H-cortisol (1.3 x 10^{-7} M) alone or ^{3}H-cortisol plus

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unlabelled cortisol (2.4×10^{-6} M) for 1 hour at 37°C. The tissue slices were then washed 5 times, and homogenized in ice cold Tris-EDTA buffer pH 7.4 containing no enzyme or 0.5 mg/ml of either trypsin, lipase, ribonuclease (RNAse) deoxyribonuclease (DNAse) or hyaluronidase (Sigma Chemical Co.). The homogenates were incubated with the enzymes at 37°C for an additional hour. Homogenates were centrifuged at 700 x g, and the supernatant fractions chromatographed on Sephadex G-200.

2) p-Chloromercuribenzoate (PCMB)

Tissue slices from four lactating cows were incubated with 2.7×10^{-9} M ^{3}H-cortisol plus ethyl alcohol or 2.7×10^{-9} M ^{3}H-cortisol plus 6.7×10^{-8} M unlabelled cortisol in Tris-EDTA buffer pH 7.4 alone (controls) or Tris-EDTA buffer pH 7.4 containing 5×10^{-3} M PCMB (Sigma Chemical), a sulfhydryl group antagonist. Tissue slices were then washed, homogenized, centrifuged and radioactivity was quantified in the 700 x g supernatant and precipitate fractions as described under routine procedures.

3) Oubain (G-Strophanthin)

Tissue slices from four lactating cows were incubated with 20 µl of 5×10^{-5} mM oubain for 30 minutes at 37°C. The tissues were further incubated for 1 hour at 37°C with 2.7×10^{-9} M ^{3}H-cortisol plus ethyl alcohol or 2.7×10^{-9} M

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^{3}H-cortisol plus 6.7 x 10^{-8} M unlabelled cortisol. Tissue slices were then washed, homogenized, centrifuged and radio-activity was quantified in the 700 x g supernatant and precipitate fractions as described under routine procedures.

Comparison of Corticoid Binding in Mammary Tissue and Blood Serum From Lactating Cows

Approximate Molecular Weight Determinations

1) Gel Filtration

Gel filtration was used to estimate the approximate molecular weight of the 700 x g mammary supernatant and serum binding components of ^{3}H-cortisol. Tissue slices from four lactating cows were incubated with 1.3 x 10^{-7} M ^{3}H-cortisol, washed, homogenized and centrifuged as previously described. A 0.6 ml sample was applied to a 1.5 x 25 cm column of Sephadex G-200 and eluted at 37°C with Tris-EDTA buffer, pH 7.4 at a flow rate of 0.25 ml/min using a hydro-static pressure head of 6 to 10 cm. Three ml fractions were collected, protein concentrations were monitored at 280 nm and radioactivity was counted from each fraction. The relative elution volume for the protein component bind-ing ^{3}H-cortisol in the 700 x g supernatant was noted.

In other experiments, whole bovine serum was incubated with 1.3 x 10^{-7} M ^{3}H-cortisol under the same experimental

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conditions as described above for mammary tissue slices. After incubation a 0.6 ml sample of serum was eluted on Sephadex G-200 as described above. The relative elution volumes for the protein components binding ^{3}H-cortisol in blood serum were noted. Protein fractions of 700 x g mammary supernatants and bovine serum which bound ^{3}H-cortisol were compared, in terms of elution volume, with a series of standard solutions which were chromatographed in the same manner. The standard solutions were Dextran 2,000, 2 x 10^6 MW; 2.0% ovalbumin, 4.3 x 10^4 MW; 2.0% β-lactoglobulin, 1.8 x 10^4 MW; and 2.0% ribonuclease A, 1.5 x 10^4 MW. The 700 x g mammary supernatant and serum binding proteins were fitted on a standard curve based upon their respective elution volumes.

2) Sucrose Density Gradient Analysis

Fresh tissue slices from two lactating cows were homogenized, and centrifuged at 700 x g for 10 minutes. The remaining supernatant fractions were then centrifuged at 45,000 x g to remove other particulate materials. The remaining supernatant was then centrifuged at 100,000 x g for two hours to isolate the cytosol fraction. Three ml of the 100,000 x g cytosol fractions were incubated with 20 µl of 1.3 x 10^{-7} M ^{3}H-cortisol for 1 hour at 37°C. This fraction was adjusted to contain 25 mg of protein/ml of cytosol.

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Bovine serum was centrifuged at 100,000 x g for 2 hours. After centrifugation, 3 ml of sera were treated with the same amount of ^{3}H-cortisol described above, and incubated under the same experimental conditions. Protein of sera was adjusted to 25 mg/ml of sera.

Samples of 0.3 ml of 100,000 x g mammary cytosol or bovine serum, previously incubated with ^{3}H-cortisol, were layered on 4.5 ml gradients of 10-30% sucrose in Tris-EDTA buffer pH 7.4 and were centrifuged at 4°C for 18 hours at 100,000 x g in a Beckman Model L-2 ultracentrifuge. The tubes were pierced after centrifugation and 32 fractions were collected directly into scintillation vials. After collection, 3 ml of ethyl alcohol was added to the vials. The vials were then counted for radioactivity as described above. The approximate sedimentation coefficients were determined by using various standards. The standards were yeast alcohol dehydrogenase (7.6 S, Sigma Chemical); liver alcohol dehydrogenase (5.0 S, Sigma Chemical); and catalase (11.0 S, Sigma Chemical). The migration of standards was estimated by optical density determinations at 280 nm.

Polyacrylamide Disc Gel Electrophoresis

Mammary tissue slices from three lactating cows were incubated with 1.3 x 10^{-7} M ^{3}H-cortisol for 1 hour at 37°C, washed five times, homogenized and 700 x g supernatant fractions were electrophoresed on 7% polyacrylamide gels

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(Appendix F). Bovine serum from three lactating cows was incubated with 1.3 x 10^{-7} M ^{3}H-cortisol then diluted 1:5 with 0.85% NaCl and electrophoresed under the same experimental conditions as the mammary 700 x g supernatants.

DEAE Cellulose Chromatography

Ion exchange chromatography (DEAE) was used as another approach to compare cortisol binding proteins in mammary tissue and blood. Thus, mammary slices from three cows were incubated for 1 hour at 37°C in 2 ml of 0.01 M potassium phosphate buffer, pH 8.0, containing 1.3 x 10^{-7} M ^{3}H-cortisol. Tissue was washed five times, homogenized and centrifuged at 700 x g, and the supernatant fluid was desalted by passage through Sephadex G-25. The fractions containing protein bound ^{3}H-cortisol were combined and applied to a DEAE cellulose column (Whatman DE-52, Whatman Co.) which had been pre-equilibrated with the 0.01 M potassium phosphate buffer, pH 8.0. The mammary cytoplasmic proteins were eluted by a stepwise 0.01 M to 2.0 M potassium phosphate gradient, pH 8.0.

Ten ml of bovine serum from three cows was dialyzed against 100 volumes of 0.01 M potassium phosphate buffer for 18 hours, then clarified by centrifugation at 2,000 x g for 10 minutes. The supernatant was incubated with 1.3 x 10^{-7} M ^{3}H-cortisol for 1 hour at 37°C and then applied to a DEAE cellulose column and eluted as described above for mammary cytoplasmic proteins.

Binding of Tritiated Cortisol,
Dexamethasone and Progesterone
to Bovine Serum

In order to determine the relative degree and order of

binding of tritiated steroids to bovine sera at 37°C, the

following experiment was carried out. Three separate tubes

containing 3 ml each of bovine serum from three cows were

incubated with 10 µl of either 2.7×10^{-9} M ^{3}H-cortisol

(48 ci/mmole), ^{3}H-dexamethasone (28 ci/mmole) or ^{3}H-proges-

terone (48 ci/mmole) for 1 hour at 37°C. After incubation,

the tubes were placed in an ice bath at 4°C and samples

containing 0.6 ml of serum plus ^{3}H-steroid and 0.4 ml of 40%

sucrose were applied to Sephadex G-200 columns. Three ml

fractions were collected, counted for radioactivity and

assayed for protein as previously described. The tubes

which contained maximal quantities of radioactive cortisol

and protein eluting in the region of CBG (MW. 52,000±1500)

were pooled. The data were expressed as µmole steroid

bound per mg protein.

Cell Free Experiments

700 x g Supernatant

Fresh unlabelled mammary tissue from four lactating

cows was homogenized and centrifuged at 700 x g. The super-

natant fluids were chromatographed on Sephadex G-25. The

fraction containing the cortisol binding receptor was

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isolated, and divided into two aliquots. One aliquot received ^{3}H-cortisol (2.7×10^{-9} M) plus ethyl alcohol while the other received ^{3}H-cortisol (2.7×10^{-9} M) plus unlabelled cortisol (6.7×10^{-8} M). The fractions were incubated for 1 hour at 37°C. After incubation, these fractions were rechromatographed on Sephadex G-25, and the quantity of ^{3}H-cortisol bound was determined. Each fraction was assayed for protein as previously described.

700 x g and 100,000 x g Precipitates

Fresh mammary tissue from four lactating cows was homogenized, and centrifuged at 700 x g. The precipitates were isolated and incubated with 5 ml of the following media: Medium 199-MEM, Tris-EDTA buffer pH 7.4, 700 x g mammary supernatant, 10% bovine serum albumin (Sigma Chemical) and 100% bovine serum. Each medium contained 1.6×10^{-9} M ^{3}H-cortisol or ^{3}H-cortisol plus 4×10^{-8} M unlabelled cortisol. The effect of unlabelled cortisol on the binding of ^{3}H-cortisol into the isolated 700 x g precipitate was determined by measuring the radioactivity in the ethyl alcohol extracts.

Mammary slices from four lactating cows were homogenized, centrifuged at 15,000 x g for 15 minutes to remove cell debris, nuclei and mitochondria. The 15,000 x g supernatant was centrifuged at 100,000 x g for 1 hour. The 100,000 x g precipitate was resuspended in Tris-EDTA buffer

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pH 7.4 using a glass hand homogenizer. The suspensions were incubated for 1 hour at 37°C with ^{3}H-cortisol (2.7×10^{-9} M) plus ethyl alcohol, or ^{3}H-cortisol plus unlabelled cortisol (6.7×10^{-8} M) or ^{3}H-cortisol plus unlabelled dexamethasone (6.7×10^{-8} M). The suspension was recentrifuged at 100,000 x g, the supernatant was discarded, the precipitate was washed once, and radioactivity was measured in the 100,000 x g precipitate.

Physiological Effects of Corticoids on Mammary Tissue Slices From Lactating Cows

A series of experiments were carried out to determine if unlabelled triamcinolone and cortexelone were capable of reducing the binding of ^{3}H-cortisol in mammary tissue slices from lactating cows. Once this was established, experiments were designed to determine the effects of several corticoids on the uptake of C^{14}-glucose into mammary tissue slices from lactating cows. These experiments were carried out in an attempt to correlate physiological activity, such as glucose uptake, with corticoid binding in the mammary tissue slice.

Triamcinolone-Cortexelone Competition

Tissue slices from four cows were incubated for 1 hour at 37°C in Medium 199:MEM containing either 2.7×10^{-9} M ^{3}H-cortisol (1, 2 ^{3}H-cortisol, 44 ci/mmole, New England

clear) or 2.7 x

thasone; 5.2 ci/

2.7×10^{-9} M ^{3}H

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Nuclear) or 2.7 x 10^{-9} M ^{3}H-dexamethasone (1, 2, 4-^{3}H-dexa-

methasone; 5.2 ci/mmole, Schwartz/Mann) plus ethyl alcohol

or 2.7 x 10^{-9} M ^{3}H-cortisol or ^{3}H-dexamethasone plus 6.7 x

10^{-8} M unlabelled triamcinolone, cortexelone (11-deoxycorti-

sol), cortisol, dexamethasone, progesterone and 17-β estra-

diol. After incubation, the tissues were homogenized,

centrifuged at 700 x g and radioactivity was determined as

described above.

C^{14}-Glucose Uptake

Tissues (500 mg) from four lactating cows were divided

into five experimental groups. Each experimental group was

further divided into five treatments. Tissues in group 1

received either 10^{-8} M of unlabelled cortexelone, cortisol,

triamcinolone dexamethasone, or progesterone. Groups 2

through 5 received either 10^{-7}, 10^{-6}, 10^{-5} or 10^{-4} M of the

five corticoids listed above or progesterone. Tissues were

incubated with unlabelled steroids for 1 hour at 37°C.

After 1 hour of incubation, the tissue samples were labelled

with 0.5 μ ci of ^{14}C-glucose and reincubated for 30 minutes

at 37°C. Controls were incubated with 20 μl ethyl alcohol

for 1 hour at 37°C, then reincubated for an additional 30

minutes with 0.5 μ ci ^{14}C-glucose. Tissues were washed 3

times in ice cold Tris-EDTA buffer pH 7.4, homogenized and

centrifuged at 700 x g. The supernatant fractions were

counted for radioactivity. Data were expressed as per cent

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inhibition of total C^{14}-glucose uptake into 700 x g super-

natants, using the ethyl alcohol controls at 0%.

In a series of preliminary experiments, tissue slices

from two lactating cows were incubated with 10^{-6} M, un-

labelled cortisol plus 10^{-4} M cortexelone for 1 hour at 37°C.

Control tissues received 20 μl of ethyl alcohol. After incu-

bation, tissues were labelled with 0.5 μ ci of ^{14}C-glucose

and reincubated for 30 minutes at 37°C. The slices were

homogenized and treated as described above.

In Vivo Experiments

Mammary Uptake of Corticoids

Six lactating Holstein cows were cannulated at the

external pudic artery and the mammary vein. Ten ml blood

samples were collected at the following intervals prior to

and after milking: -30, -15, -6, 0, +6, +12, +16, +20, +30

and 60 minutes. Arterial and venous blood samples were

assayed for total glucocorticoids, as described by Smith

et al. (1972, 1973) and Appendix G. Data were expressed as

ng/ml differences, between arterial and venous samples.

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STATISTICAL ANALYSES

Statistical analyses of hormone competition experiments were carried out using a two-way analysis of variance with individual animals as blocks and hormones as treatments. Dunnet's "T" test (Dunnett, 1955) was used to compare control means (^{3}H-cortisol or ^{3}H-dexamethasone plus ethyl alcohol) with individual treatment means (^{3}H-cortisol or ^{3}H-dexamethasone plus unlabelled hormones). Two component curve analyses were done on Scatchard plots using the method of least squares regression. The data were then transformed to produce a single "best fitting" linear regression line representing the high affinity (specific) corticoid binding component (Appendix D). Statistical analysis of regression line slopes (Kd's) and x intercepts (number of molecules of corticoid bound per mammary cell) from animals in various physiological states were performed using Scheffe's test for multiple comparisons (Appendix H).

A Student's "t" test was used for comparing means of ^{3}H-cortisol bound to components within 700, 15,000 and 100,000 x g mammary supernatants after chromatography on Sephadex G-200 columns.

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Corticoid binding and C^{14} glucose uptake were correlated by standard product-moment procedures (Sokol and Rohlf, 1969). The significance of treatments (i.e., milking time) in A-V difference experiments was examined by applying the Student's "t" statistic on the null hypothesis: Ho = $\overline{A-V}$ = 0, at each interval of milking. All analyses were performed with the aid of a CDC 6500 computer and an Olivetti programable calculator. Scatchard plots were obtained from a CDC calcomp plotter.

RESULTS

Hormone Binding

Preliminary Competition Experiments

Unless otherwise stated, mammary tissue slices were
incubated with hormones, and the binding subsequently
determined in 700 x g supernatant or precipitate fractions.
In the absence of unlabelled cortisol, uptake of ^{3}H-cortisol
(DPM/μg total DNA) was 35 to 63 times greater in 700 x g
supernatants than in 700 x g precipitates of mammary tissue
(Table 1). Addition of unlabelled cortisol significantly
reduced ($p < 0.05$) the uptake of ^{3}H-cortisol in 700 x g
supernatant and precipitate fractions, but quantitatively
this reduction ranged from only 5-17% in the supernatant and
30-35% in the precipitate fractions. Surface area of mam-
mary slices did not alter the degree of competition between
^{3}H-cortisol and unlabelled cortisol; thus, subsequent ex-
periments used eight whole tissue slices averaging 0.2-0.4
mm^3 per tube as described in routine procedures.

Table 1. Binding of ^{3}H-cortisol to 700 x g supernatant and precipitate
fractions of mammary tissue slices from lactating cows[a]

	DPM/μg Total DNA[c]			
	Whole	Halved	Quartered	Minced
	———— 700 x g supernatant ————			
^{3}H-cortisol + ethyl alcohol	227	223	270	153
^{3}H-cortisol + cortisol[b]	188	211	255	135
	———— 700 x g precipitate ————			
^{3}H-cortisol + ethyl alcohol	4.3	4.6	5.0	5.8
^{3}H-cortisol + cortisol[b]	2.9	3.2	3.5	3.8

[a]Approximately 0.5 g tissue was suspended in 3 ml MEM-199 medium pH 7.4.
^{3}H-cortisol concentration was 2.7×10^{-9} M in ethyl alcohol. Unlabelled
cortisol concentration was 6.7×10^{-8} M in ethyl alcohol.

[b]All values significantly less than respective ethyl alcohol controls
($P < 0.05$).

[c]Total DNA was measured in 700 x g precipitate after ethyl alcohol
extraction of ^{3}H-corticoids, and was used to adjust data in 700 x g
supernatant and precipitate fractions.

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Specificity of Corticoid Binding in Mammary Tissue Slices from Lactating Cows

1) Competition Experiments

Uptake of ^{3}H-cortisol (DPM/μg total DNA), in the absence of unlabelled cortisol, was 61 times greater in 700 x g supernatants than in 700 x g precipitates (Table 2). When compared with ethyl alcohol controls, unlabelled cortisol and dexamethasone reduced the binding of ^{3}H-cortisol in 700 x g supernatant fractions by 7.8 ($p < 0.05$) and 33.6% ($p < 0.01$), respectively; and by 47.4 ($p < 0.01$) and 57.9% ($p < 0.01$), respectively, in 700 x g precipitates (Table 2). In contrast, progesterone, 17β-estradiol and testosterone did not significantly ($p < 0.05$) reduce binding of ^{3}H-cortisol in either fraction.

When ^{3}H-dexamethasone was used in place of ^{3}H-cortisol and the various unlabelled hormones were added as described previously, cortisol and dexamethasone reduced the binding of ^{3}H-dexamethasone in 700 x g supernatants by 10.9 and 10.3% ($p < 0.05$), respectively; and by 27.5 and 50.0% ($p < 0.01$), respectively in 700 x g precipitates (Table 2). Again, the other unlabelled hormones were without significant effect on reducing ^{3}H-dexamethasone binding. Uptake of ^{3}H-dexamethasone, in the absence of unlabelled dexamethasone, was 39 times greater in 700 x g supernatants than in 700 x g precipitates (Table 2).

Table 2. Hormonal inhibition of ^{3}H-cortisol and ^{3}H-dexamethasone binding to mammary tissue slices from lactating cows.[a]

Unlabelled hormone	^{3}H-cortisol DPM/μg total DNA[d]		^{3}H-dexamethasone DPM/μg total DNA[d]	
	700 x g supernatant	700 x g precipitate	700 x g supernatant	700 x g precipitate
Cortisol	107[b]	1.0[c]	138[b]	2.9[c]
Dexamethasone	77[c]	0.8[c]	139[b]	2.0[c]
Progesterone	111	1.7	158	3.7
17β estradiol	115	1.8	154	3.5
Testosterone	133	1.9	161	3.7
Ethyl alcohol control	116	1.9	155	4.0

[a] Concentration of unlabelled steroids was 6.7×10^{-8} M in ethyl alcohol, while the concentration of 3H-cortisol and 3H-dexamethasone was 2.7×10^{-9} M in ethyl alcohol.

[b] Less than ethyl alcohol control (P < 0.05).

[c] Less than ethyl alcohol control (P < 0.01).

[d] Total DNA was measured in 700 x g precipitate after ethyl alcohol extraction of 3H-corticoids, and was used to adjust data in 700 x g supernatant and precipitate fractions.

2) Scatchard Analysis

Scatchard plots obtained after incubation of mammary tissue slices from lactating cows with various levels of ^{3}H-cortisol-cortisol at 37°C are given in Figures 1A and 1B. The 700 x g supernatant and precipitate fractions of mammary tissue each had two components with affinity for cortisol. One component had a high affinity for cortisol, with a dissociation constant Kd = 5.0 x 10^{-10} M, as calculated from the slope, in 700 x g supernatant and 0.39 x 10^{-10} M in 700 x g precipitate fractions. These components were saturable at low hormone concentrations and believed to represent specifically bound cortisol. Figures 1A and 1B illustrate saturation of the high affinity binding component as the concentration of ^{3}H-cortisol was increased. The number of molecules of cortisol bound per cell by the high affinity component, as calculated from the intercept at the X axis (Figures 1A and 1B) was estimated to be 2837 in 700 x g supernatant and 25 to 700 x g precipitate fractions. The second component had almost zero slope (Figure 1A, and 1B), had low affinity for cortisol and appeared unsaturable. For example, this component bound cortisol at concentrations up to 4 x 10^{-8} M. Binding of cortisol to this component was also nonspecific, since ^{3}H-cortisol binding could not be eliminated by a fivefold excess of unlabelled cortisol.

Figure 1A.   Scatchard plot of ^{3}H-cortisol binding (0.005 to 4 x 10^{-8} M) at 37°C to 700 x g supernatant fractions of mammary tissue slices from lactating cows. The x's represent a mean value [$\frac{Bound}{Free}$ vs Bound] of quadruplicate samples for a particular cow at a specific concentration of hormone. Curve analyses were done using the method of least squares regression.

700Xg MAMMARY SUPERNATANT

$Kd \simeq 5.0 \times 10^{-10} M$

Bound (Moles / μg DNA x 10^{-17})

Bound/Free (Liters / μg DNA x 10^{-9})
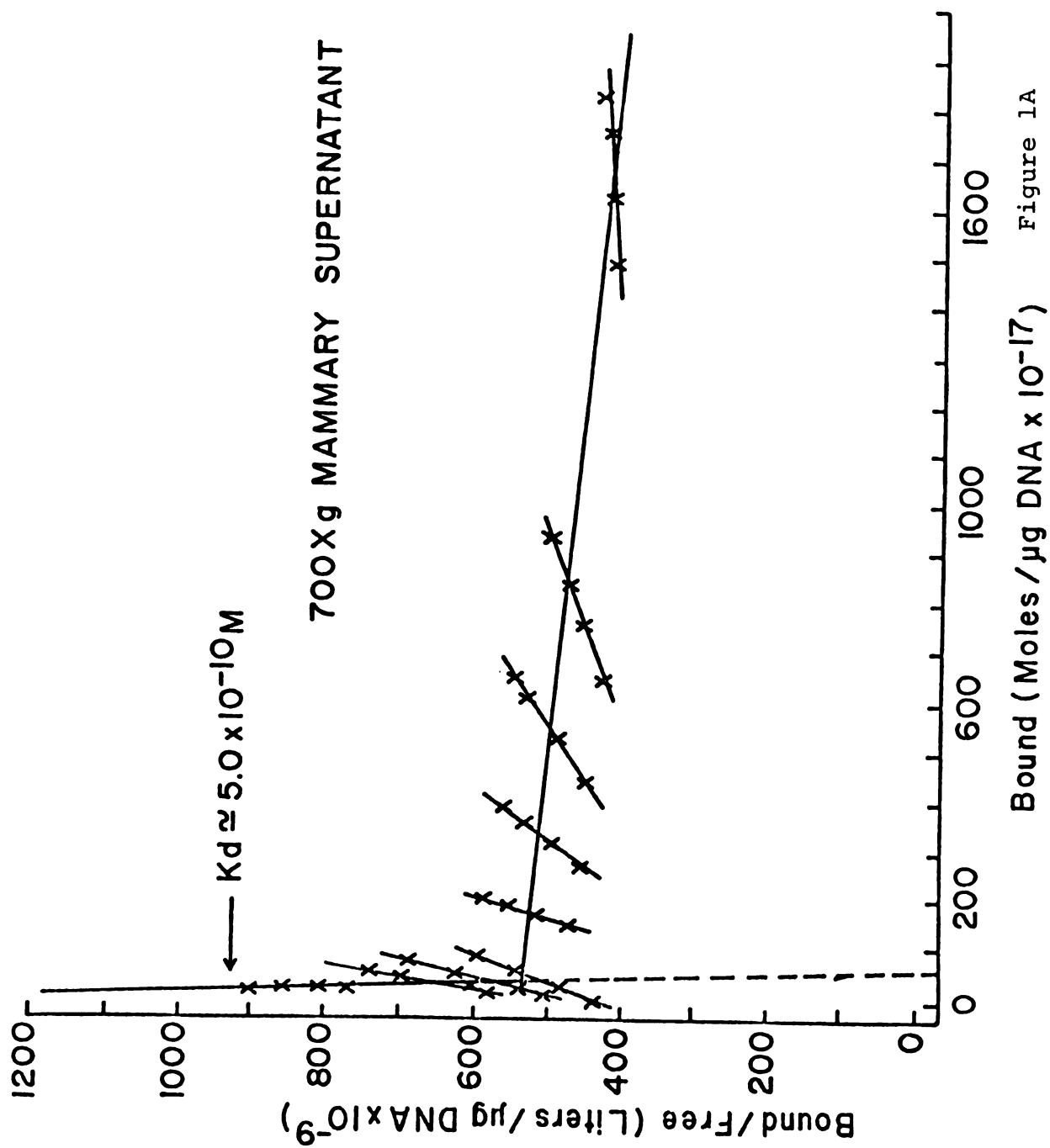
Figure 1A

Figure 1B. Scatchard plot of ^{3}H-cortisol binding (0.005 to 4 x 10^{-8} M) at 37°C to 700 x g precipitate fractions of mammary tissue slices from lactating cows. The x's represent a mean value [$\frac{Bound}{Free}$ vs Bound] of quadruplicate samples for a particular cow at a specific concentration of hormone. Curve analyses were done using the method of least squares regression.

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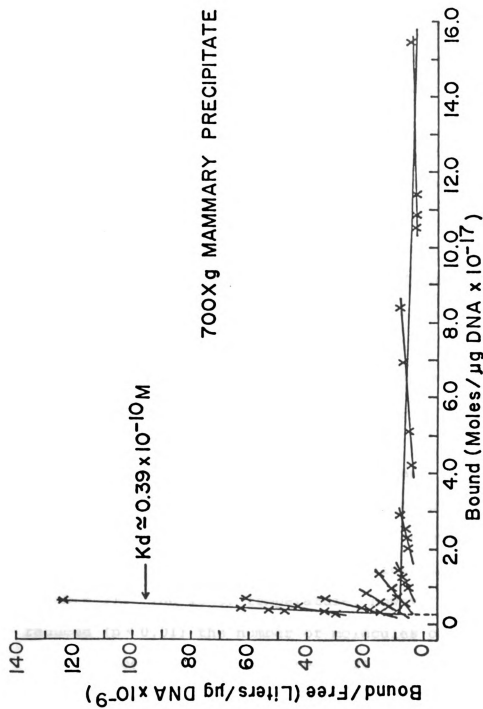
Figure 1B

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Figures 2A and 2B show the specific high affinity bind-
ing components for cortisol at 37°C in 700 x g supernatant
and precipitate fractions of mammary tissue from lactating
cows after transformation and adjustment for nonspecific
binding (Appendix D). After adjustment and transformation,
the Kd for cortisol was 10.5 x 10^{-10} M in 700 x g super-
natant and 0.5 x 10^{-10} M in 700 x g precipitate fractions.
The adjusted high affinity components bound 1277 molecules
of cortisol per cell in 700 x g supernatant fractions and
22 molecules of cortisol in 700 x g precipitate fractions.
Transformation of Scatchard data did not significantly
($p > 0.05$) affect the dissociation constants for cortisol in
both tissue fractions, but significantly ($p < 0.05$) reduced
the total number of molecules of cortisol bound per cell
by approximately 55% when compared with the values obtained
with non-transformed data.

The dissociation constants from adjusted and unadjusted
Scatchard plots, of cortisol bound to mammary tissue slices
from lactating cows incubated at 4°C, were not significantly
different ($p > 0.05$) from those for tissue slices incubated
at 37°C (Table 3). The lower temperature, however, signifi-
cantly reduced ($p < 0.01$) the number of molecules of cortisol
bound in mammary cells by approximately 82% (calculated from
adjusted values).

Figure 2A. Transformed Scatchard plot of ^{3}H-cortisol bind-
ing (0.005 to 0.041 x 10^{-8} M) at 37°C to 700 x
g supernatant fractions of mammary tissue
slices from lactating cows. The Δ's represent
means [$\frac{\text{Bound}}{\text{Free}}$ (corrected) vs Bound] of quadrupli-
cate samples for a particular cow at a specific
concentration of hormone. Curve analyses were
done using the method of least squares regres-
sion.

Bound/Free (Liters/µg DNA x 10^{-9})

360
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280
240
200
160
120
80
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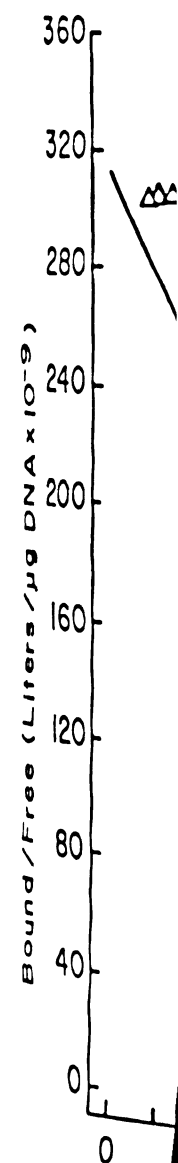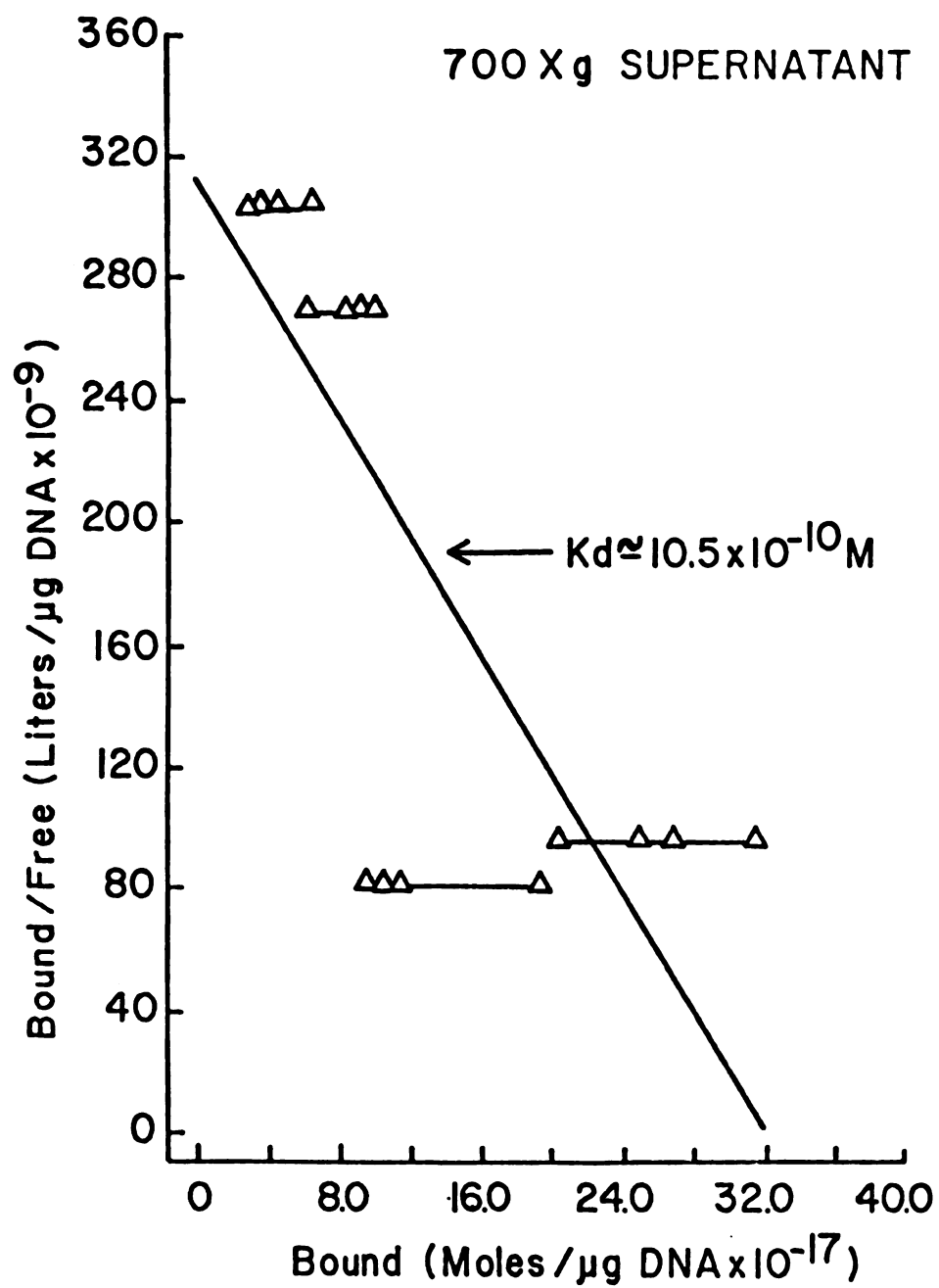
Figure 2A

Figure 2B. Transformed Scatchard plot of ^{3}H-cortisol bind-
ing (0.005 to 0.041 x 10^{-8} M) at 37°C to 700 x
g precipitate fractions of mammary tissue slices
from lactating cows. The Δ's represent means
[$\frac{Bound}{Free}$ (corrected) vs Bound] of quadruplicate
samples for a particular cow at a specific
concentration of hormone. Curve analyses were
done using the method of least squares regres-
sion.

Bound/Free (Liters/μg DNA x10^{-9})

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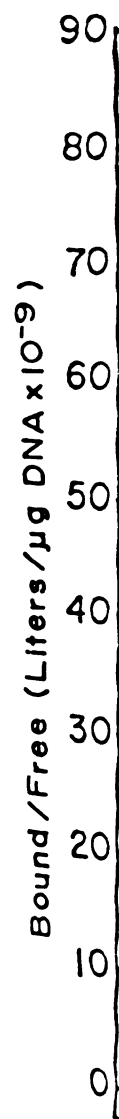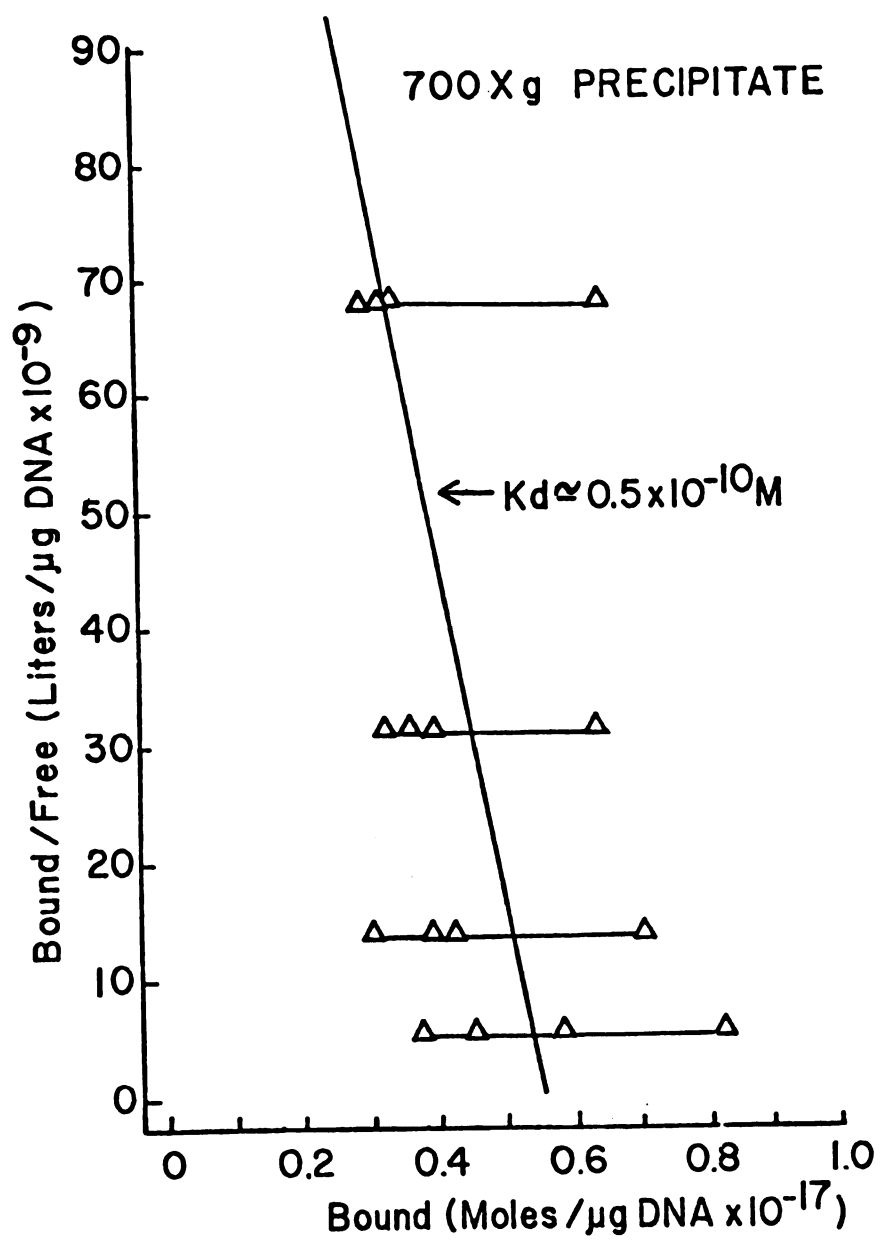
Figure 2B

Table 3. Summary of Scatchard plots of cortisol binding to mammary tissue slices from lactating cows at 4°C.a

Kd (dissociation constant) $\times 10^{-10}$ M cortisol	Number of molecules of cortisol bound in mammary cell ± standard error of mean

Table 3. Summary of Scatchard plots of cortisol binding to mammary tissue slices from lactating cows at 4°C.[a]

	Kd (dissociation constant) x 10^{-10} M cortisol		Number of molecules of cortisol bound in mammary cell ± standard error of mean	
	700 x g		700 x g	
	Supernatant	Precipitate	Supernatant	Precipitate
Adjusted	16.6[c]	0.26[c]	133 ± 13[b]	6 ± 4[b]
Unadjusted	14.7	0.20	1275 ± 36	6 ± 3

[a]Tissue slices were incubated at 4°C with 0.005 to 4 x 10^{-8} M of a combination of ^{3}H-cortisol and unlabelled cortisol. Statistical analysis was performed on adjusted values using Scheffe's test for multiple comparisons (Appendix H).

[b]Adjusted means are statistically lower than values obtained from lactating tissue slices incubated at 37°C

[c]Adjusted means are not significantly different (p > 0.05) from values obtained from lactating tissue slices incubated at 37°C.

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Scatchard plots of dexamethasone bound to mammary tissue slices from lactating cows at 37°C also revealed a single high affinity binding component, which was specific for dexamethasone and an unsaturable nonspecific dexamethasone binding component in both 700 x g supernatant and precipitate tissue fractions. Table 4 summarizes the data from Scatchard plots of dexamethasone treated tissues. The dissociation constants were not significantly different from those of cortisol in 700 x g supernatant fractions of mammary tissue slices from lactating cows (Figures 1A and 2A). Dissociation constants for dexamethasone-receptor complexes in 700 x g precipitate fractions of mammary tissue slices from lactating cows were significantly greater ($p < 0.01$) than those for cortisol (Figures 1B and 2B). Mammary tissue slices bound significantly more ($p < 0.01$) molecules of dexamethasone in mammary cells than cortisol. Mammary tissue slices bound approximately twice as many molecules of dexamethasone per mammary cell than cortisol (Table 4).

Corticoid Binding in Mammary Tissue Slices from Virgin Heifers, 1-Month Prepartum, Lactating and Dry Cows

1) Competition Experiments

Virgin Heifers

Unlabelled cortisol and dexamethasone reduced the binding of ^{3}H-cortisol in 700 x g supernatant fractions by 11.7 ($p < 0.05$) and 15.6% ($p < 0.05$), respectively and by 43

Table 4. Summary of Scatchard plots of dexamethasone binding to mammary tissue slices from lactating cows at 37°C.a

Kd (dissociation constant) $\times 10^{-10}$ M dexamethasone	Number of molecules of dexamethasone bound in mammary cell $\pm$ SE of mean

Table 4. Summary of Scatchard plots of dexamethasone binding to mammary tissue slices from lactating cows at 37°C.[a]

	Kd (dissociation constant) $\times 10^{-10}$ M dexamethasone		Number of molecules of dexamethasone bound in mammary cell $\pm$ SE of mean	
	700 x g		700 x g	
	Supernatant	Precipitate	Supernatant	Precipitate
Adjusted	12.8[c]	4.6[b]	1789 $\pm$ 22[b]	168 $\pm$ 19[b]
Unadjusted	13.2	4.3	3575 $\pm$ 86	242 $\pm$ 32

[a]Tissue slices were incubated at 37°C with 0.005 to 4 $\times 10^{-8}$ M of a combination of ^{3}H-dexamethasone and unlabelled dexamethasone. Statistical analysis was performed on adjusted values using Scheffe's test for multiple comparisons (Appendix H).

[b]Adjusted means are statistically greater ($p < 0.01$) than values obtained from lactating tissue slices incubated at 37°C with cortisol.

[c]Adjusted means are not significantly different ($p > 0.05$) from values obtained from lactating tissue slices incubated at 37°C with cortisol.

(p < 0.01) and 62.2% (p < 0.01), respectively, in 700 x g pre-
cipitates (Table 5). Progesterone, 17β-estradiol and testo-
sterone did not significantly (p > 0.05) reduce binding of
^{3}H-cortisol in either fraction. In the absence of unlabelled
cortisol, uptake of ^{3}H-cortisol (DPM/μg total DNA) was 21
times greater in 700 x g supernatants than precipitates
(Table 5). In experiments where ^{3}H-dexamethasone was used
in place of ^{3}H-cortisol, unlabelled cortisol and dexametha-
sone reduced binding by 12.7 (p < 0.05) and 27% (p < 0.01),
respectively, in 700 x g supernatants. Unlabelled dexametha-
sone reduced ^{3}H-dexamethasone binding by 75% (p < 0.01) in
700 x g precipitates (Table 5). Unlabelled progesterone,
17β-estradiol and testosterone were without effect on
^{3}H-dexamethasone binding. The uptake of ^{3}H-dexamethazone,
in the absence of unlabelled hormones, was only 5.7 times
greater in 700 x g supernatants than precipitates (Table 5).

1-Month Prepartum Cows

Unlabelled cortisol and dexamethasone reduced the bind-
ing of ^{3}H-cortisol in 700 x g supernatant fractions 22.1%
(p < 0.01) and 19.8% (p < 0.05), respectively, and by 55.2
(p < 0.01) and 65.5% (p < 0.01), respectively, in 700 x g pre-
cipitates (Table 6). In experiments where ^{3}H-dexamethasone
was used in place of ^{3}H-cortisol, unlabelled cortisol and
dexamethasone reduced the binding of ^{3}H-dexamethasone in
700 x g supernatants by 17.4 (p < 0.05) and 19.0% (p < 0.05)

	^3H-cortisol DPM/µg Total DNA[d]	^3H-dexamethasone DPM/µg Total DNA[d]

Table 5. Hormonal inhibition of ^{3}H-cortisol and ^{3}H-dexamethasone binding to mammary tissue slices from virgin heifers.[a]

Unlabelled hormone	^{3}H-cortisol DPM/μg Total DNA[d]		^{3}H-dexamethasone DPM/μg Total DNA[d]	
	700 x g supernatant	700 x g precipitate	700 x g supernatant	700 x g precipitate
Cortisol	68[b]	2.1[c]	55[b]	10.9
Dexamethasone	65[b]	1.4[c]	46[c]	2.8[c]
Progesterone	80	3.4	58	11.7
17β-estradiol	79	3.7	57	14.2
Testosterone	77	3.7	71	12.8
Ethyl alcohol control	77	3.7	63	11.1

[a]Concentration of unlabelled steroids was 6.7×10^{-8} M in ethyl alcohol, while the concentration of ^{3}H-cortisol and ^{3}H-dexamethasone was 2.7×10^{-9} M in ethyl alcohol.

[b]Less than ethyl alcohol control ($p < 0.05$).

[c]Less than ethyl alcohol control ($p < 0.01$).

[d]Total DNA was measured in 700 x g precipitate after ethyl alcohol extraction of ^{3}H-corticoids, and was used to adjust data in 700 x g supernatant and precipitate fractions.

Table 6. Hormonal inhibition of ^{3}H-cortisol and ^{3}H-dexamethasone binding to mammary tissue slices from 1-month prepartum cows.[a]

^{3}H-cortisol DPM/µg Total DNA[d]	^{3}H-dexamethasone DPM/µg Total DNA[d]

Table 6. Hormonal inhibition of ^{3}H-cortisol and ^{3}H-dexamethasone binding to mammary tissue slices from 1-month prepartum cows.[a]

Unlabelled hormone	^{3}H-cortisol DPM/μg Total DNA[d]		^{3}H-dexamethasone DPM/μg Total DNA[d]	
	700 x g supernatant	700 x g precipitate	700 x g supernatant	700 x g precipitate
Cortisol	67[c]	1.3[c]	100[b]	4.4[b]
Dexamethasone	69[b]	1.0[c]	98[b]	2.6[c]
Progesterone	84	2.8	108	4.6
17β-estradiol	90	2.7	120	6.1
Testosterone	84	2.9	116	4.9
Ethyl alcohol control	86	2.9	121	5.2

[a]Concentration of unlabelled steroids was 6.7×10^{-8} M in ethyl alcohol, while the concentration of ^{3}H-cortisol and ^{3}H-dexamethasone was 2.7×10^{-9} M in ethyl alcohol.

[b]Less than ethyl alcohol control ($p < 0.05$).

[c]Less than ethyl alcohol control ($p < 0.01$).

[d]Total DNA was measured in 700 x g precipitate after ethyl alcohol extraction of ^{3}H-corticoids, and was used to adjust data in 700 x g supernatant and precipitate fractions.

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respectively, and by 15.4 (p < 0.05) and 50% (p < 0.01), respectively, in 700 x g precipitates (Table 6). Unlabelled progesterone, 17β-estradiol and testosterone did not significantly (p > 0.05) reduce binding of either ^{3}H-cortisol or ^{3}H-dexamethasone in mammary slices from 1-month prepartum cows (Table 6). Uptake of ^{3}H-cortisol and ^{3}H-dexamethasone, in the absence of unlabelled hormones, was 23 and 29 times greater respectively in 700 x g supernatants than precipitates.

Lactating Cows

Unlabelled cortisol and dexamethasone reduced binding of ^{3}H-cortisol in 700 x g supernatant fractions by 17.4 (p < 0.05) and 20.4% (p < 0.01), respectively, and by 50.0 (p < 0.01) and 55% (p < 0.01), respectively, in 700 x g precipitates (Table 7). As previously shown, unlabelled progesterone, 17β-estradiol and testosterone did not significantly (p > 0.05) reduce the binding of ^{3}H-cortisol in either fraction of lactating tissue slices. The uptake of ^{3}H-cortisol in the absence of unlabelled hormones was 52 times greater in 700 x g supernatants than precipitates.

Cortisol and dexamethasone reduced the binding of ^{3}H-dexamethasone in 700 x g supernatants by 9.7% (p < 0.05) and by 44 (p < 0.01) and 70% (p < 0.01), respectively, in 700 x g precipitates (Table 7). The other unlabelled steroids failed to significantly (p > 0.05) reduce ^{3}H-dexamethasone

^{3}H-cortisol
DPM/µg Total DNA[d]

^{3}H-dexamethasone
DPM/µg Total DNA[d]

Table 7. Hormonal inhibition of ^{3}H-cortisol and ^{3}H-dexamethasone binding to mammary tissue slices from lactating cows.[a]

Unlabelled hormone	^{3}H-cortisol DPM/µg Total DNA[d]		^{3}H-dexamethasone DPM/µg Total DNA[d]	
	700 x g supernatant	700 x g precipitate	700 x g supernatant	700 x g precipitate
Cortisol	81[b]	0.95[c]	131[b]	4.7[c]
Dexamethasone	78[c]	0.86[c]	131[b]	2.5[c]
Progesterone	95	1.8	122	5.9
17B-estradiol	93	2.2	135	6.0
Testosterone	96	2.0	130	6.2
Ethyl alcohol control	98	1.9	145	8.4

[a] Concentration of unlabelled steroids was 6.7×10^{-8} M in ethyl alcohol, while the concentration of ^{3}H-cortisol and dexamethasone was 2.7×10^{-9} in ethyl alcohol.

[b] Less than ethyl alcohol control ($p < 0.05$).

[c] Less than ethyl alcohol control ($p < 0.01$).

[d] Total DNA was measured in 700 x g precipitate after ethyl alcohol extraction of ^{3}H-corticoids, and was used to adjust data in 700 x g supernatant and precipitate fractions.

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binding. The uptake of ^{3}H-dexamethasone, in the absence of unlabelled hormones, was 17 times greater in 700 x g super-natants than precipitates.

Dry Cows

Competitive hormone binding experiments carried out on mammary tissue slices from dry cows showed that unlabelled cortisol and dexamethasone reduced the uptake of ^{3}H-cortisol 22% ($p < 0.01$) and 13.6% ($p < 0.05$) in 700 x g supernatant fractions (Table 8). Unlabelled cortisol and dexamethasone also reduced the uptake of ^{3}H-cortisol by 43 ($p < 0.01$) and 62.2% ($p < 0.01$), respectively, in 700 x g precipitates (Table 8). When ^{3}H-dexamethasone was substituted for ^{3}H-cortisol, unlabelled cortisol did not significantly ($p > 0.05$) reduce the binding of ^{3}H-dexamethasone in 700 x g supernatant fractions. Unlabelled dexamethasone reduced ^{3}H-dexamethasone binding by 17.9% ($p < 0.05$) in 700 x g supernatant fractions. Unlabelled cortisol and dexamethasone, however, reduced ^{3}H-dexamethasone binding by 40.2 ($p < 0.01$) and 77% ($p < 0.01$), respectively, in 700 x g precipitate fractions. Unlabelled progesterone, 17β-estradiol and testosterone failed to reduce ^{3}H-cortisol and ^{3}H-dexamethasone binding in mammary tissue fractions from dry cows (Table 8). Uptake of ^{3}H-cortisol and ^{3}H-dexamethasone, in the absence of unlabelled hormones was 22 and 6 times greater, respectively, in 700 x g supernatants than precipitates.

Table 8. Hormonal inhibition of ^{3}H-cortisol and ^{3}H-dexamethasone binding to mammary tissue slices from dry cows (nonpregnant, nonlactating).[a]

Unlabelled hormone	^{3}H-cortisol DPM/μg Total DNA[d]		^{3}H-dexamethasone DPM/μg Total DNA[d]	
	700 x g supernatant	700 x g precipitate	700 x g supernatant	700 x g precipitate
Cortisol	69[c]	2.1[c]	65	6.7[c]
Dexamethasone	76[b]	1.4[c]	55[b]	2.6[c]
Progesterone	94	3.4	65	11.0
17β-estradiol	94	4.1	70	10.8
Testosterone	89	3.7	63	16.4
Ethyl alcohol control	88	3.7	67	11.2

[a] Concentration of unlabelled steroids was 6.7×10^{-8} M in ethyl alcohol, while the concentration of ^{3}H-cortisol and ^{3}H-dexamethasone was 2.7×10^{-9} M in ethyl alcohol.

[b] Less than ethyl alcohol control ($p < 0.05$).

[c] Less than ethyl alcohol control ($p < 0.01$).

[d] Total DNA was measured in 700 x g precipitate after ethyl alcohol extraction of ^{3}H-corticoids, and was used to adjust data in 700 x g supernatant and precipitate fractions.

2) Scatchard Analysis: Virgin Heifers,
   1-Month Prepartum, Lactating and Dry
   Cows (nonpregnant and nonlactating)

A summary of the results obtained from adjusted and un-
adjusted Scatchard plots using ^{3}H-cortisol and ^{3}H-dexametha-
sone in mammary tissue slices from virgin heifers, 1-month
prepartum, lactating and dry cows are given in Tables 9-12.
Scatchard plots of hormone bound in the various physiologi-
cal states displayed two components which bound cortisol
and dexamethasone in 700 x g supernatant and precipitate
fractions. One component had high affinity for corticoid
and was saturated at low hormone concentrations. This com-
ponent was believed to represent the specific binding
component for corticoids. The other component had low affin-
ity for corticoid and was practically unsaturable. This
component was thought to be nonspecific for corticoids.
Mammary tissue slices from lactating cows, virgin heifers,
1-month prepartum and dry cows, incubated at 37°C with
various concentrations of ^{3}H-cortisol bound this hormone
with high affinity (Kd = 0.30 x 10^{-10} to 20 x 10^{-10} M,
Table 9). The Kd for the cortisol-receptor complex in 700
x g supernatant fractions of virgin heifers, was signifi-
cantly (p < 0.05) greater when compared with those for
1-month prepartum, lactating and dry cows (Table 9).
Furthermore, the Kd for the cortisol-receptor complex in
700 x g mammary supernatant fractions of 1-month prepartum

Table 9. Summary of Scatchard plot dissociation constants (Kd's) for cortisol binding to mammary tissue slices from virgin heifers, 1-month prepartum, lactating and dry (nonpregnant, nonlactating) cows.[a]

| Physiological State | Cortisol Kd (dissociation constant) $\times 10^{-10}$ M | | | |
| | 700 x g Supernatant | | Precipitate | |
	(adj)	(unadj)	(adj)	(unadj)
Virgin Heifers	20^b	17	2.4^b	2.5
1-Month Prepartum Cows	3.8^c	6.1	0.92^c	1.1
Lactating Cows	10.4^d	5.5	0.30^c	0.40
Dry (nonpregnant, nonlactating) cows	7.8^d	6.5	2.3^b	1.8

(adj) adjusted values, obtained from Scatchard plots corrected for nonspecific binding.

(unadj) unadjusted values, obtained from Scatchard plots uncorrected for nonspecific binding.

[a] Statistical analysis was performed on adjusted values using Scheffe's test for multiple comparisons (Appendix H).

[b,c,d] Adjusted means in the same column, sharing the same superscripts are not significantly different (i.e., $p > 0.05$). All others in the same column differ significantly ($p < 0.05$).

cows was significantly ($p < 0.05$) lower when compared with those for lactating and dry cows (Table 9). Dissociation constants for cortisol-receptor complexes in 700 x g mammary supernatant fractions of lactating and dry cows were not significantly ($p > 0.05$) different from one another (Table 9).

The Kd's for cortisol-receptor complexes in 700 x g precipitate fractions of virgin heifers and dry cows were significantly ($p < 0.05$) greater than those for 1-month pre-partum and lactating cows (Table 9). There was no signifi-cant ($p > 0.05$) difference between the cortisol Kd's of 700 x g precipitates of virgin and dry and between 1-month pre-partum and lactating cows (Table 9).

Statistical analyses of the number of cortisol mole-cules bound in mammary cells showed that virgin heifers and dry cows bound significantly ($p < 0.05$) fewer molecules of cortisol in 700 x g supernatant fractions when compared with 1-month prepartum cows and lactating cows (Table 10). But there was no significant difference ($p > 0.05$) between the number of molecules of cortisol bound in virgin heifers and dry cows (Table 10). Lactating cows bound significantly ($p < 0.05$) more molecules of cortisol in 700 x g supernatant fractions than 1-month prepartum cows (Table 10).

There was no significant ($p > 0.05$) difference between animals of various physiological states with regard to the numbers of cortisol molecules bound in 700 x g precipitate fractions (Table 10).

Table 10. Summary of Scatchard plot x-intercepts (number of molecules of cortisol bound in mammary cell $\pm$ SE of mean) of mammary tissue slices from virgin heifers, 1-month prepartum, lactating and dry (nonpregnant, nonlactating) cows.[a]

Physiological State	Number of molecules of cortisol bound in mammary cell $\pm$ SE 700 x g			
	Supernatant		Precipitate	
	(adj)	(unadj)	(adj)	(unadj)
Virgin Heifers	387 $\pm$ 17[b]	2187 $\pm$ 64	26 $\pm$ 9[b]	36 $\pm$ 18
1-Month Prepartum Cows	520 $\pm$ 22	1289 $\pm$ 36	22 $\pm$ 12[b]	28 $\pm$ 8
Lactating Cows	1242 $\pm$ 48	2221 $\pm$ 71	21 $\pm$ 10[b]	27 $\pm$ 6
Dry (nonpregnant, nonlactating) Cows	307 $\pm$ 18[b]	1044 $\pm$ 48	29 $\pm$ 6[b]	36 $\pm$ 10

(adj) adjusted values, obtained from Scatchard plots corrected for nonspecific binding.

(unadj) unadjusted values, obtained from Scatchard plots uncorrected for nonspecific binding.

[a] Statistical analysis was performed on adjusted values using Scheffe's test for multiple comparisons (Appendix H).

[b] Adjusted means in the same column, sharing the same superscripts are not significantly different (i.e., $p > 0.05$). All others in the same column differ significantly ($p < 0.05$).

The Kd for the dexamethasone-receptor complex, in 700 x g mammary supernatant fractions of virgin heifers was significantly ($p < 0.05$) lower when compared with that for lactating and dry cows (Table 11). There was no significant ($p > 0.05$) difference between dexamethasone-receptor Kd's in 700 x g mammary supernatant fractions of lactating and dry cows (Table 11). Dissociation constants for dexamethasone-receptor complexes in 700 x g precipitate fractions did not significantly differ with regard to physiological state (Table 11).

Statistical comparisons of the number of dexamethasone molecules bound in mammary tissue slices showed a significant difference between animals of various physiological states (Table 12). For example, virgin heifers and dry cows bound significantly ($p < 0.05$) fewer molecules of dexamethasone in 700 x g supernatant fractions when compared with lactating cows (Table 12). There was no significant difference between the number of molecules of dexamethasone bound in 700 x g supernatants of virgin heifers and dry cows (Table 12).

There was no significant difference between the number of dexamethasone molecules bound in 700 x g precipitate fractions of mammary slices from virgin heifers and lactating cows (Table 12). However, 700 x g precipitate fractions from virgin mammary slices bound significantly ($p < 0.05$)

Table 11. Summary of Scatchard plot dissociation constants (Kd's) for dexamethasone binding to mammary tissue slices from virgin heifers, lactating and dry (nonpregnant, non-lactating) cows.[a]

Physiological State	Dexamethasone Kd (dissociation constant) $\times 10^{-10}$ M 700 x g			
	Supernatant		Precipitate	
	(adj)	(unadj)	(adj)	(unadj)
Virgin Heifers	5.9[b]	5.9	5.7[c]	5.6
Lactating Cows	13.1[c]	12.9	4.4[c]	4.4
Dry (nonpregnant, nonlactating) Cows	9.2[c]	2.4	6.4[c]	1.3

(adj) adjusted values, obtained from Scatchard plots corrected for nonspecific binding.

(unadj) unadjusted values, obtained from Scatchard plots uncorrected for nonspecific binding.

[a] Statistical analysis was performed on adjusted values using Scheffe's test for multiple comparisons (Appendix H).

[b,c] Adjusted means in the same column, sharing the same superscripts are not significantly different (i.e., $p > 0.05$). All others in the same column differ significantly ($p < 0.05$).

Table 12. Summary of Scatchard plot x-intercepts (number of molecules of dexamethasone bound in mammary cell ± SE of mean) of mammary tissue slices from virgin heifers, lactating and dry (nonpregnant, nonlactating) cows.[a]

Physiological State	Number of molecules of dexamethasone bound in mammary cell ± SE			
	700 x g			
	Supernatant		Precipitate	
	(adj)	(unadj)	(adj)	(unadj)
Virgin Heifers	422 ± 12^{b}	1235 ± 46	229 ± 23^{b}	370 ± 26
Lactating Cows	1778 ± 61^{c}	6452 ± 122	177 ± 19^{b}	290 ± 19
Dry (nonpregnant, nonlactating) Cows	407 ± 22^{b}	1178 ± 64	129 ± 26^{c}	212 ± 18

(adj) adjusted values, obtained from Scatchard plots corrected for nonspecific binding.

(unadj) unadjusted values, obtained from Scatchard plots uncorrected for nonspecific binding.

[a] Statistical analysis was performed on adjusted values using Scheffe's test for multiple comparisons (Appendix H).

[b,c] Adjusted means in the same column sharing the same superscripts are not significantly different (i.e., p > 0.05). All others in the same column differ significantly (p < 0.05).

more molecules of dexamethasone when compared with dry cows
(Table 12). Lactating cows bound significantly ($p < 0.05$)
more molecules of dexamethasone in 700 x g precipitates than
dry cows (Table 12).

Tables 13 and 14 show the statistical comparisons be-
tween cortisol and dexamethasone Scatchard plot slopes (Kd's)
and x-intercepts (numbers of molecules of corticoid bound in
mammary cell) in mammary slices from animals in various
physiological states. The Kd for the cortisol-receptor
complex in 700 x g supernatant fractions of virgin mammary
tissue slices was significantly ($p < 0.01$) greater than that
for dexamethasone (Table 13). However, the Kd for the
cortisol-receptor complex in 700 x g precipitate fractions
of virgin mammary slices was significantly ($p < 0.05$) less
than that for dexamethasone (Table 13). There was no sig-
nificant ($p > 0.05$) difference between the cortisol and dexa-
methasone Kd's of 700 x g supernatant fractions of mammary
tissue slices from lactating or dry cows (Table 13). In
contrast, the Kd for cortisol in 700 x g precipitate frac-
tions of mammary slices from lactating cows was significantly
($p < 0.01$) less than that for dexamethasone (Table 13). The
cortisol-receptor complex Kd for cortisol in 700 x g precipi-
tate fractions of dry cows was significantly ($p < 0.05$) less
than that for dexamethasone (Table 13).

There was no significant ($p > 0.05$) difference between
the number of cortisol and dexamethasone molecules bound in

Table 13. Statistical comparison of Scatchard plot slopes (Kd's) for cortisol and dexamethasone in mammary slices from animals in various physiological states.[a]

Physiological State	Kd (dissociation constant) x 10^{-10} M			
	700 x g			
	Supernatant		Precipitate	
	Cort[b]	Dex[b]	Cort	Dex
Virgin Heifers	20[c]	5.9	2.4[d]	5.7
Lactating Cows	10.4[e]	13.1	0.30[c]	4.4
Dry (nonpregnant, nonlactating) Cows	7.8[e]	9.2	2.3[d]	6.4

[a]Statistical analysis was performed on adjusted values using Scheffe's test for multiple comparisons (Appendix H).

[b]Cort = cortisol, Dex = dexamethasone.

[c]Significantly different from value for dexamethasone ($p < 0.01$).

[d]Significantly different from value for dexamethasone ($p < 0.05$).

[e]No significant difference between values for cortisol and dexamethasone (i.e., $p > 0.05$).

Table 14. Statistical comparison of Scatchard plot x-intercepts (number of molecules of corticoid bound in mammary cell) for cortisol and dexamethasone in mammary slices from animals in various physiological states.[a]

Physiological State	Number of molecules of corticoid bound in mammary cell ± SE 700 x g			
	Supernatant		Precipitate	
	Cort[b]	Dex[b]	Cort	Dex
Virgin Heifers	385 ± 64[e]	422 ± 11	26 ± 9[c]	299 ± 23
Lactating Cows	1242 ± 48[d]	1778 ± 61	21 ± 10[c]	177 ± 19
Dry (nonpregnant, nonlactating) Cows	307 ± 18[e]	407 ± 22	29 ± 6[c]	129 ± 25

[a] Statistical analysis was performed on adjusted values using Scheffe's test for multiple comparisons (Appendix H).

[b] Cort = cortisol, Dex = dexamethasone.

[c] Significantly different from value for dexamethasone (p < 0.01).

[d] Significantly different from value for dexamethasone (p < 0.05).

[e] No significant difference between values for cortisol and dexamethasone (i.e., p > 0.05).

the 700 x g supernatant fractions of virgin heifers and dry cows (Table 14). Mammary tissue from lactating cows, however, bound significantly (p < 0.05) more molecules of dexamethasone than cortisol in 700 x g supernatant fractions (Table 14). Tissue slices from cattle in all physiological states studied, bound significantly (p < 0.01) more molecules of dexamethasone in 700 x g precipitate fractions than cortisol (Table 14). Tissue slices from all animals studied bound approximately twice as many total molecules of dexamethasone (number of molecules bound in 700 x g supernatant plus the number of molecules bound in 700 x g precipitates) than cortisol (Table 14).

Metabolism of ^{3}H-cortisol

The majority of radioactivity in the bound and free fractions of 700 x g supernatants, isolated by Sephadex G-200 chromatography, migrated in a spot on thin-layer chromatograms which superimposed on standard cortisol (Figure 3). No metabolites of ^{3}H-cortisol were found in the 700 x g supernatant fractions.

Figure 3. Thin-layer chromatography of ^{3}H-cortisol extracted from "protein-bound" and "free" fractions of 700 x g supernatants of mammary tissue slices from lactating cows.
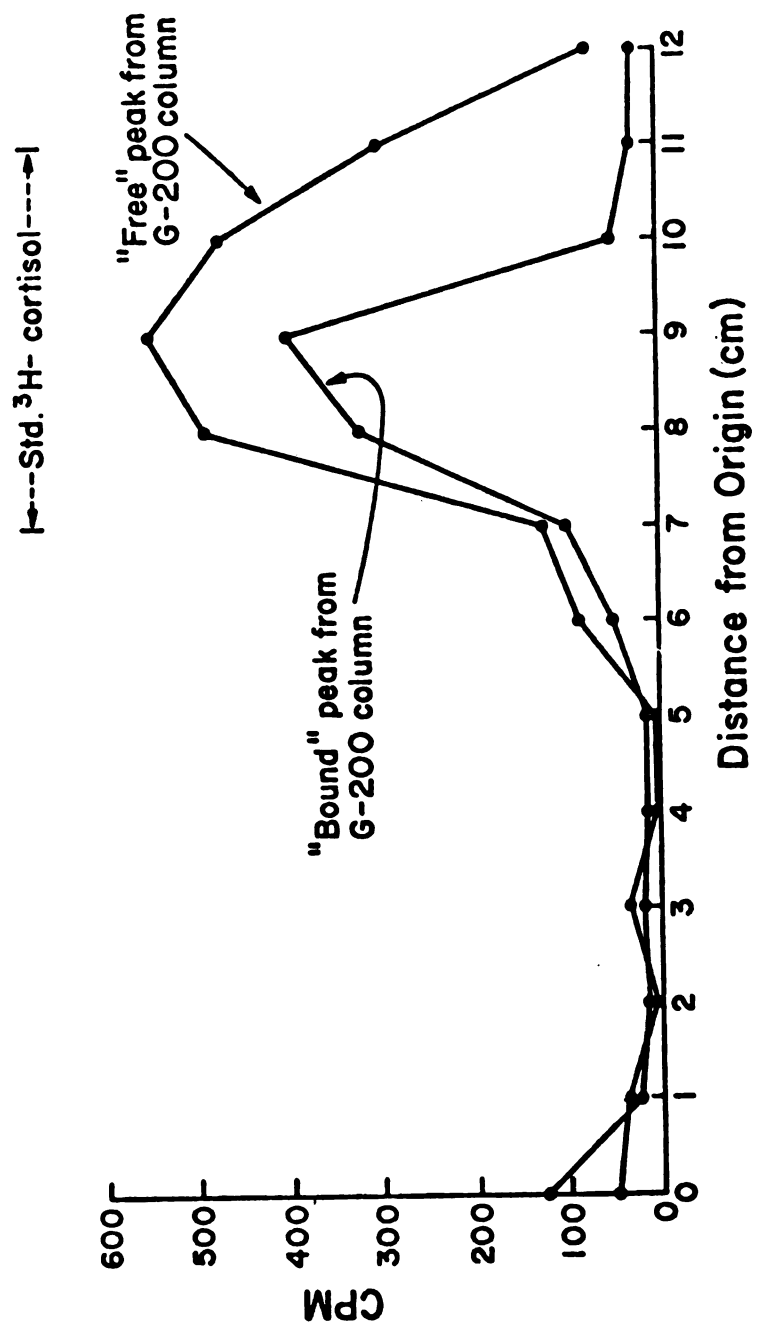
Figure 3

Physicochemical Characterization of Corticoid
Receptors in Mammary Tissue
From Lactating Cows

Gel Filtration Chromatography

1) Mammary Cytosol Fractions

700 x g Supernatant

Two radioactive peaks were present in the Sephadex
G—25 elution profiles of 700 x g supernatant samples obtained
from mammary tissue slices treated with either ^{3}H-cortisol
plus ethyl alcohol or ^{3}H-cortisol plus unlabelled cortisol
(Figure 4). The peak of ^{3}H-cortisol radioactivity in frac-
tion number 5 was associated with a major protein peak from
the 700 x g supernatant fractions. The peak of radioactiv-
ity found in fraction 12 had no corresponding peak of
assayable protein. Addition of unlabelled cortisol reduced
the quantity of radioactivity present in the protein-rich
fraction (number 5) by approximately 66%, when compared
with ^{3}H-cortisol-ethyl alcohol controls.

15,000 and 100,000 x g Supernatants

The profiles of radioactivity (not shown) for the 700,
15,000 or 100,000 x g supernatants after Sephadex G-200
chromatography were similar to those shown in Figure 4 for
[3]H-cortisol plus ethyl alcohol. The amount of ^{3}H-cortisol
associated with the proteins of 700, 15,000 and 100,000 x g
supernatants were comparable and averaged 3,400 $\pm$ 134,

Figure 4. Gel filtration (Sephadex G-25) elution profile

of 700 x g mammary supernatant from tissue

slices of lactating cows treated with ^{3}H-cortisol

plus ethyl alcohol or ^{3}H-cortisol plus unlabelled

cortisol.
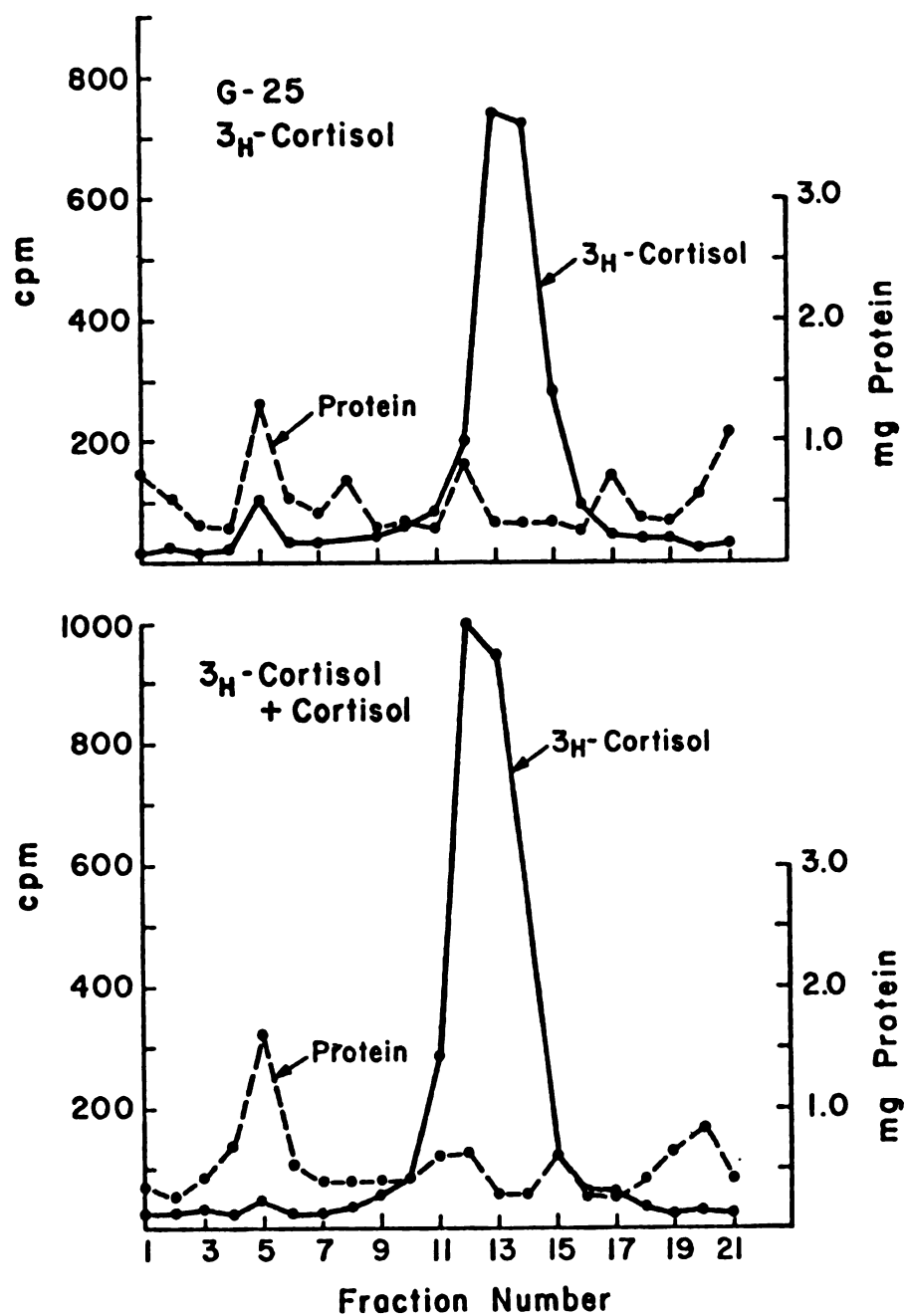
Figure 4

4,900 ± 146 and 3,900 ± 138 cpm/mg protein, respectively. These mean values were not significantly different (p < 0.05) from one another which suggested that experimental use of either of these tissue supernatants would be representative of ^{3}H-cortisol binding within mammary cytosol.

2) Mammary Nuclear Fractions

700 x g Precipitate

The Sephadex G-25 elution profiles of KCl extracts of 700 x g precipitate fractions of mammary tissue slices from lactating cows labelled with ^{3}H-cortisol plus ethyl alcohol and ^{3}H-cortisol plus unlabelled cortisol are shown in Figure 5. Tritiated cortisol was associated with proteins which eluted at fraction 7 (Figure 5). Radioactive corti-sol in fraction 7 was reduced by 67%, with the addition of unlabelled cortisol (Figure 5). A large quantity of ^{3}H-cortisol remained unbound in the nuclear extract (fractions 10 and 21). Unbound cortisol had no associated protein peak.

Potential Binding Inhibitors

1) Enzymes

Trypsin markedly reduced the ^{3}H-cortisol bound in the fraction associated with protein after chromatography of 700 x g supernatants on Sephadex G-200 (Table 15). The reduction in binding was three times greater than that

Figure 5. Gel filtration (Sephadex G-25) elution profile

of 0.3 M KCl extract of 700 x g mammary pre-

cipitate, isolated from tissues incubated with

^{3}H-cortisol plus ethyl alcohol or ^{3}H-cortisol

plus unlabelled cortisol.
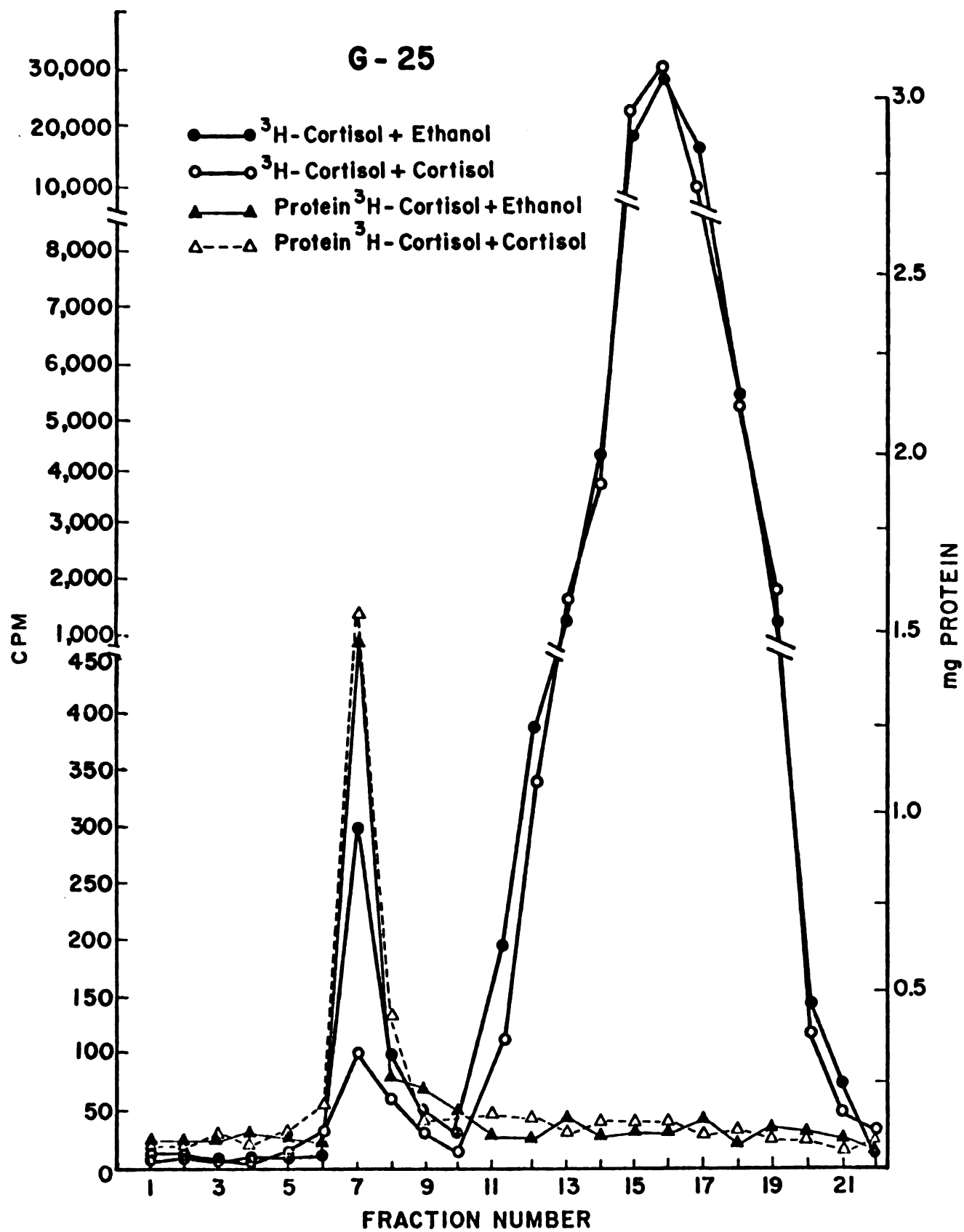
Figure 5

Table 15. Enzymatic inhibition of ^{3}H-cortisol binding to 700 x g super-
natant protein fractions of mammary tissue slices from
lactating cows.[a]

Treatment	CPM/ml[b]
^{3}H-cortisol + ethyl alcohol	450
^{3}H-cortisol + trypsin	50
^{3}H-cortisol + RNase	550
^{3}H-cortisol + DNase	440
^{3}N-cortisol + lipase	400
^{3}H-cortisol + hyaluronidase	425
^{3}H-cortisol + cortisol	150

[a] Concentration of ^{3}H-cortisol was 1.3×10^{-7} M. Unlabelled cortisol had
a concentration of 2.4×10^{-6} M. The respective enzyme concentrations
were 0.5 mg/ml of Tris-EDTA buffer, pH 7.4.

[b] Data expressed in CPM/ml, are mean values for peaks of ^{3}H-cortisol
bound to macromolecular components from Sephadex G-200 columns.

obtained with unlabelled cortisol. Lipase, RNase, DNase or hyaluronidase did not affect binding of ^{3}H-cortisol in the protein-rich fraction of the 700 x g supernatant. A large quantity of ^{3}H-cortisol remained unbound (not associated with protein). The ^{3}H-cortisol associated with these fractions was not altered by enzymes.

2) p-Chloromercuribenzoate (PCMB)

Unlabelled cortisol significantly reduced binding of ^{3}H-cortisol in 700 x g supernatant and precipitate fractions as expected in control tissue slices (Table 16). However, PCMB-treated slices unexpectedly bound more ^{3}H-cortisol in the presence of unlabelled cortisol.

3) Oubain (G-Strophanthin)

Oubain (10^{-5} mM) had no significant effect on the uptake or binding of ^{3}H-cortisol in either 700 x g supernatant or precipitate fractions of mammary tissue slices from lactating cows. Unlabelled cortisol significantly ($p < 0.05$) reduced the binding of ^{3}H-cortisol in 700 x g supernatant and precipitate fractions of tissues which were either pretreated with oubain or received no oubain pretreatment, when compared with controls which received ethyl alcohol and ^{3}H-cortisol (not shown).

Table 16. Effects of p-chloromercuribenzoate on ^{3}H-cortisol binding to mammary tissue slices from lactating cows.[a]

	DPM/μg Total DNA[d]	
	^{3}H-cortisol + ethyl alcohol	^{3}H-cortisol + cortisol
Control		
700 x g supernatant	225	212[b]
700 x g precipitate	4.9	3.0[b]
p-Chloromercuribenzoate		
700 x g supernatant	236	333[c]
700 x g precipitate	2.8	2.9

[a] PCMB concentration was 5×10^{-3} M. The concentrations of ^{3}H-cortisol and unlabelled cortisol were 2.7×10^{-9} M and 6.7×10^{-8} M, respectively, in ethyl alcohol.

[b] Less than respective ethyl alcohol control ($p < 0.05$).

[c] Greater than respective ethyl alcohol control ($p < 0.05$).

[d] Total DNA was measured in 700 x g precipitate after ethyl alcohol extraction of ^{3}H-corticoids, and was used to adjust data in 700 x g supernatant and precipitate fractions.

Comparison of Corticoid Binding in
Mammary Tissue and Blood Serum
From Lactating Cows

Approximate Molecular Weight Determinations

1) Gel Filtration

Approximate molecular weights of the major ^{3}H-cortisol

binding proteins in 700 x g mammary supernatants and bovine

serum were approximately 3 x 10^6 and 6 x 10^4, respectively

(Figure 6). In some experiments, there was a second minor

protein component in serum which bound ^{3}H-cortisol and

eluted with the void volume; this may represent nonspecific

^{3}H-cortisol binding to immune globulins or other high mole-

cular weight proteins. It should be noted that these esti-

mates are only an approximate indication of the order of

magnitude of the molecular weights.

2) Sucrose Density Gradient Analysis

Additional attempts were made to compare the approxi-

mate molecular weights of the proteins which bound ^{3}H-

cortisol in mammary cytosol and blood serum using sucrose

gradient centrifugation.

The mammary cytosol binding component (MCBC) which was

complexed with ^{3}H-cortisol sedimented in the 11.0S region

of sucrose gradients (Figure 7). This suggested that the

molecular weight of the mammary cytosol receptor was approx-

imately 250,000 or greater, since it sedimented in the

Figure 6. Gel filtration (Sephadex G-200) elution volumes of 700 x g supernatant, mammary cortisol binding proteins, serum cortisol binding proteins and various standard proteins of known molecular weight.
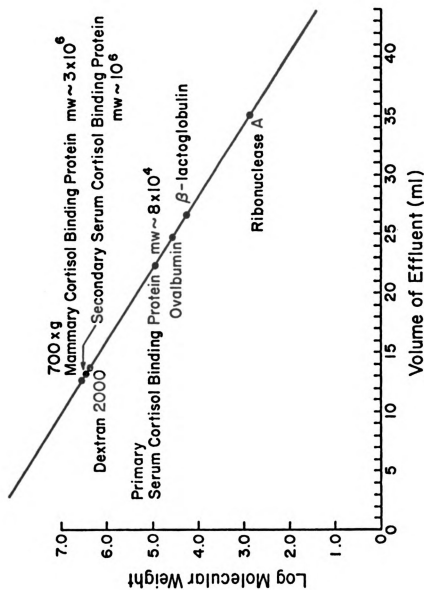
Figure 6

Figure 7. Sucrose density gradiant patterns of ^{3}H-cortisol binding
to 100,000 x g mammary supernatant fractions and bovine
sera. Yeast alcohol dehydrogenase (YAD), mw = 150,000;
liver alcohol dehydrogenase (LAD), mw = 83,000; and
catalase, mw = 250,000 were used as standards to determine
approximate sedimentation coefficients and molecular
weights. MCBC represents the mammary cytosol binding com-
ponent and SBC represents the serum binding component for
cortisol.
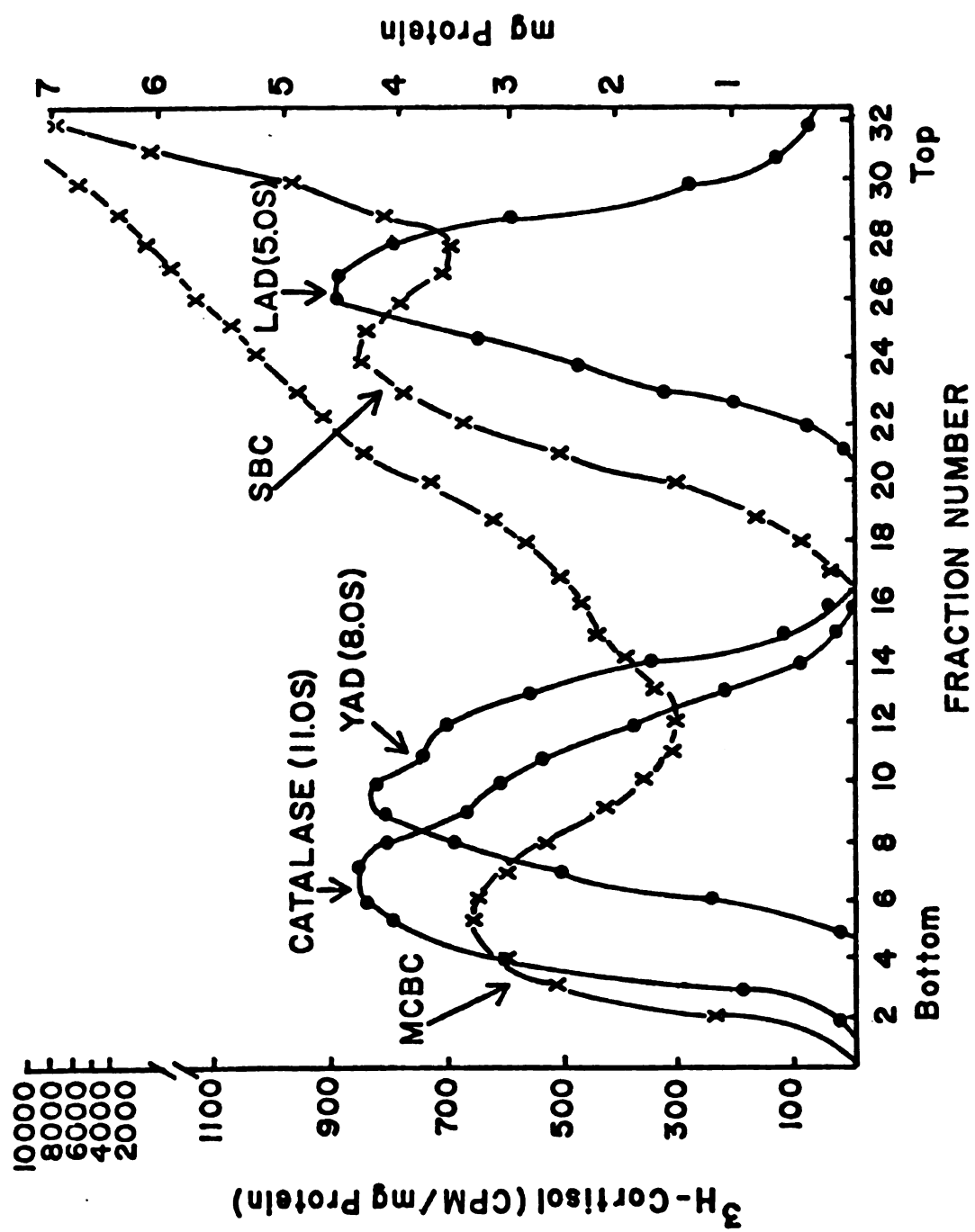
Figure 7

region of catalase, which has a molecular weight of 250,000. In contrast, the serum binding component (SBC) which also bound ^{3}H-cortisol sedimented in the 5.0S region of the sucrose gradient. This suggested that the approximate molecular weight of the SBC was approximately 80,000 or greater since it sedimented in the region of liver alcohol dehydrogenase (LAD) which has a molecular weight of 83,000.

Polyacrylamide Disc Gel Electrophoresis

As shown in Figure 8, the majority of radioactivity in the 700 x g mammary supernatants after electrophoresis was associated with proteins which migrated 2.5-3.0 cm from the gel origin. In contrast, bovine serum-bound ^{3}H-cortisol (Figure 9) was primarily associated with proteins which migrated 5.0-6.0 cm from the origin. These data further suggested that the major cortisol binding proteins of serum were not identical with the major binding proteins of mammary cytoplasm.

DEAE Cellulose Chromatography

Results of ion exchange chromatography experiments showed that ^{3}H-cortisol, bound to mammary cytoplasmic proteins, eluted with 0.3 M potassium phosphate (Figure 10), whereas ^{3}H-cortisol bound to bovine serum proteins eluted with 0.05 and 0.1 M potassium phosphate (Figure 11). These data provided additional evidence that the proteins binding

Figure 8. Polyacrylamide disc-gel electrophoretic profile of ^{3}H-cortisol binding to protein in 700 x g supernatant fractions of mammary tissue slices from lactating cows.
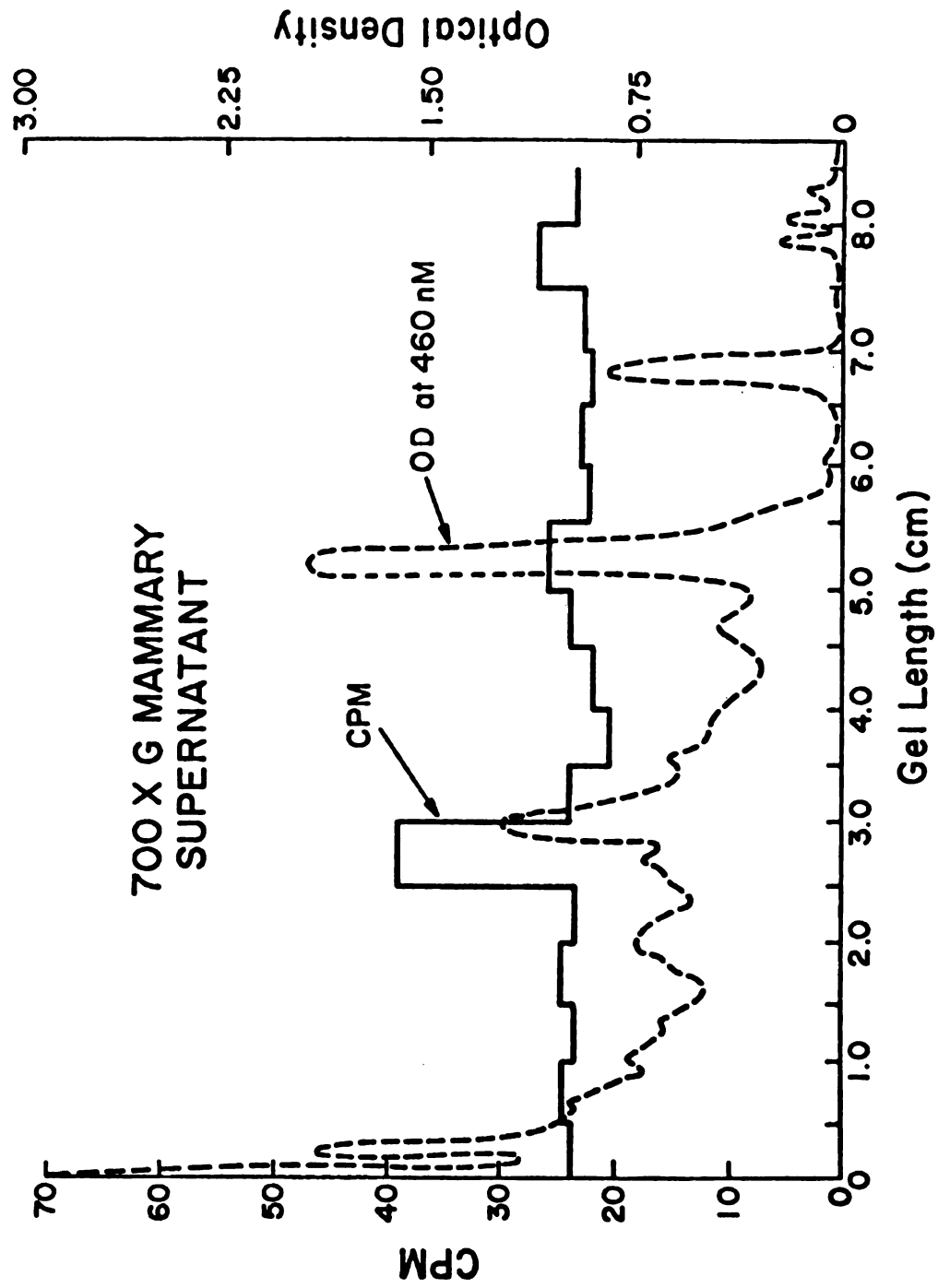
Figure 8

Figure 9. Polyacrylamide disc-gel electrophoretic profile of ^{3}H-cortisol binding to proteins of bovine sera.
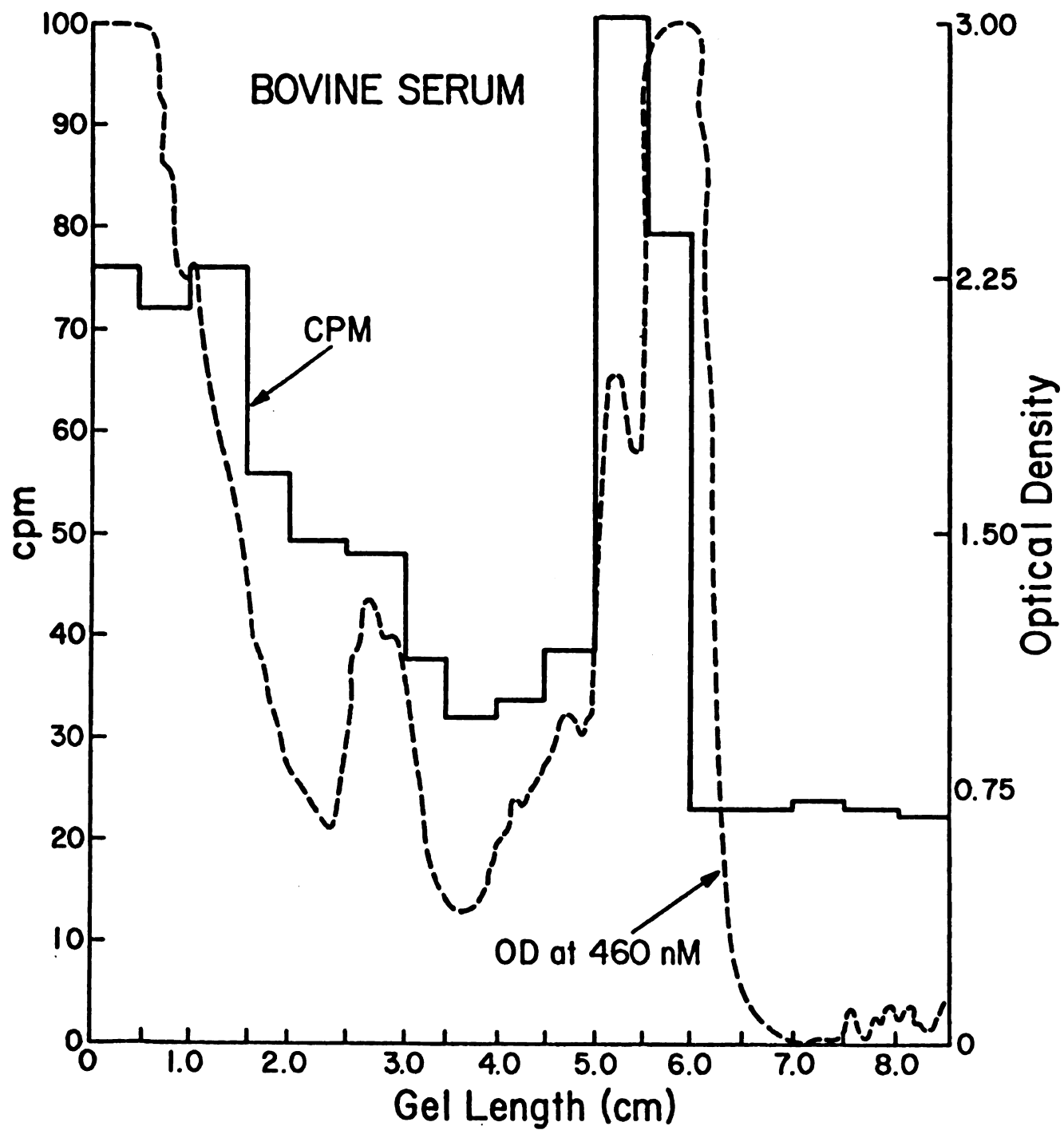
Figure 9

Figure 10. DEAE cellulose elution profile of ^{3}H-cortisol binding to 700 x g supernatant of mammary tissue slices from lactating cows. Protein elution was carried out using a stepwise-discontinuous gradient of 0.01, 0.05, 0.1, 0.3 and 2.0 M potassium phosphate, pH 8.0. Protein was monitored at 280 nm.
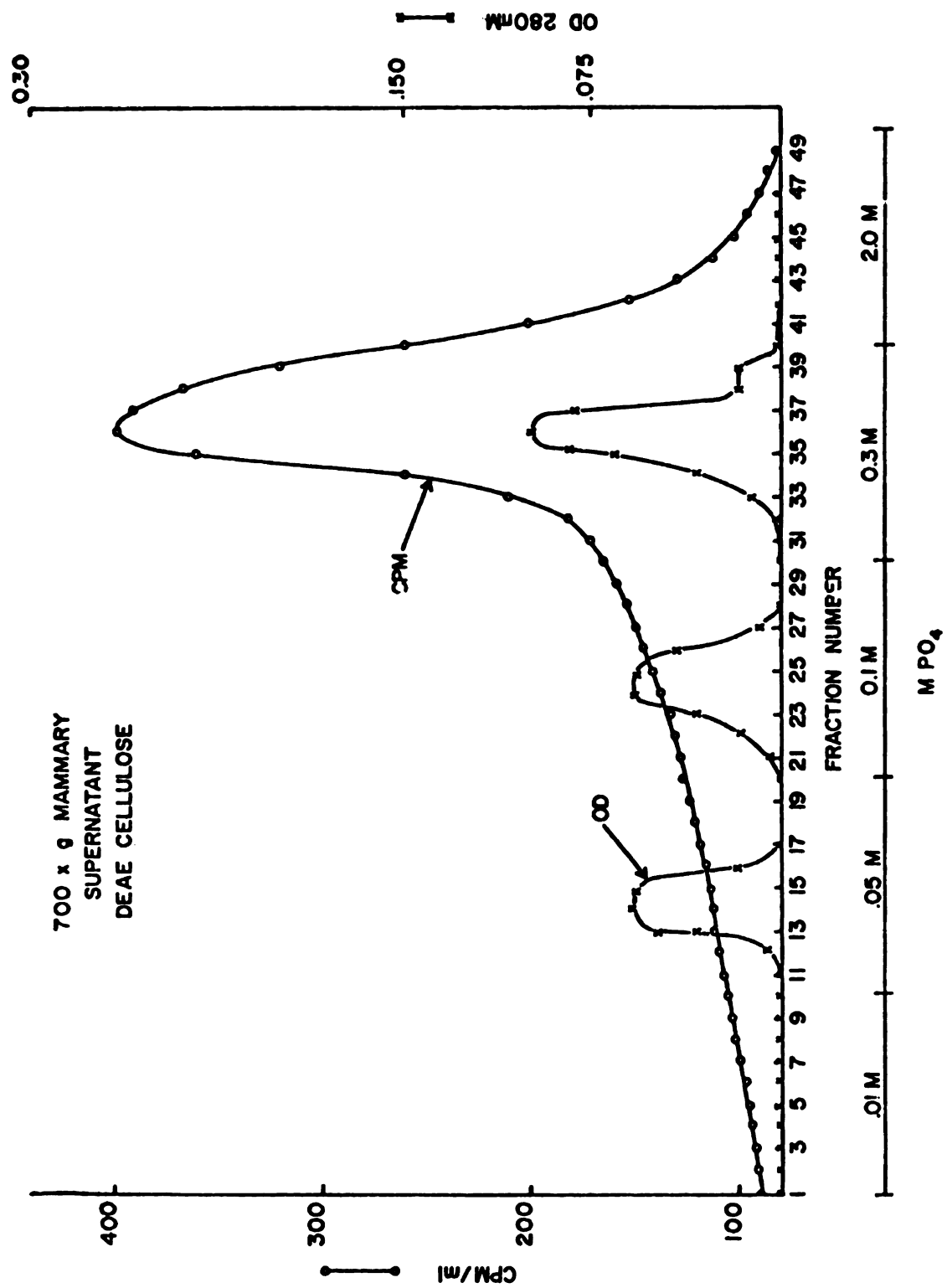
Figure 10

Figure 11. DEAE cellulose elution profile of ^{3}H-cortisol binding to bovine sera. Protein elution was carried out using a stepwise-discontinuous gradient of 0.01, 0.05, 0.1, 0.3 and 2.0 M potassium phosphate, pH 8.0. Protein was monitored at 280 nm.
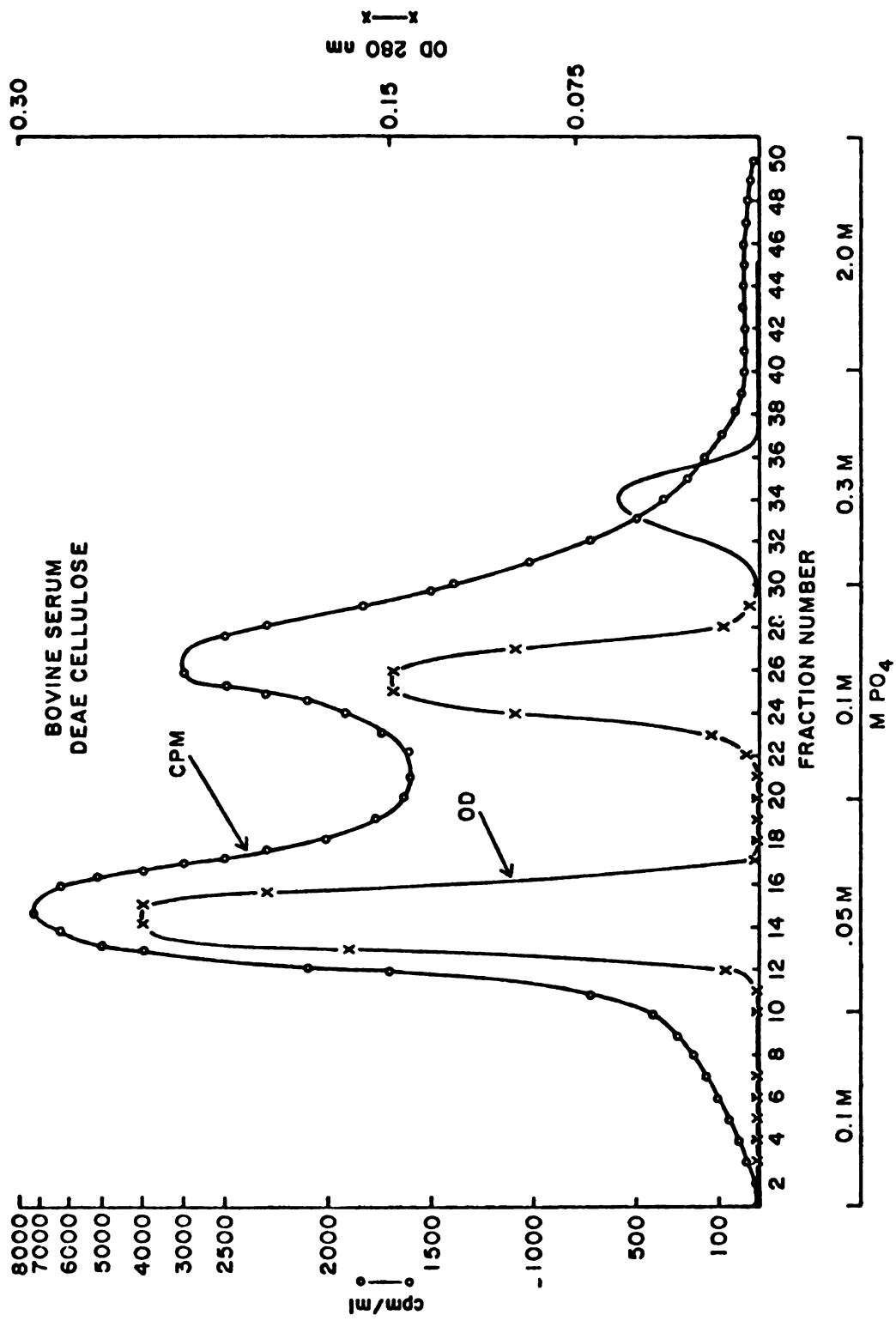
Figure 11

^{3}H-cortisol in mammary cytosol and bovine serum were dissimilar.

Binding of Tritiated Cortisol, Dexamethasone and Progesterone to Bovine Serum

In experiments designed to determine the relative degree and order of binding of tritiated steroids to bovine sera at 37°C, sera bound 3.23×10^{-13} μ moles of ^{3}H-cortisol, 2.19×10^{-13} μ moles of ^{3}H-progesterone and 0.599×10^{-13} μ moles of ^{3}H-dexamethasone per mg of protein.

Cell-Free Experiments

700 x g Supernatant

Cortisol binding protein(s) from 700 x g supernatant fractions of fresh mammary tissue slices from lactating cows were isolated by Sephadex G-25 chromatography. The isolated cortisol binding protein(s) were then incubated directly with ^{3}H-cortisol plus ethyl alcohol and ^{3}H-cortisol plus unlabelled cortisol and rechromatographed. These proteins bound 93 cpm ^{3}H-cortisol/mg protein (Figure 12). Addition of unlabelled cortisol reduced the protein bound ^{3}H-cortisol in the cell-free isolate by 11%. A large quantity of unbound ^{3}H-cortisol was associated with fractions 8 to 18 (Figure 12).

Figure 12. Sephadex G-25 elution profile of 700 x g mammary cortisol receptor protein(s), after cell-free incubation with ^{3}H-cortisol or ^{3}H-cortisol plus unlabelled cortisol.
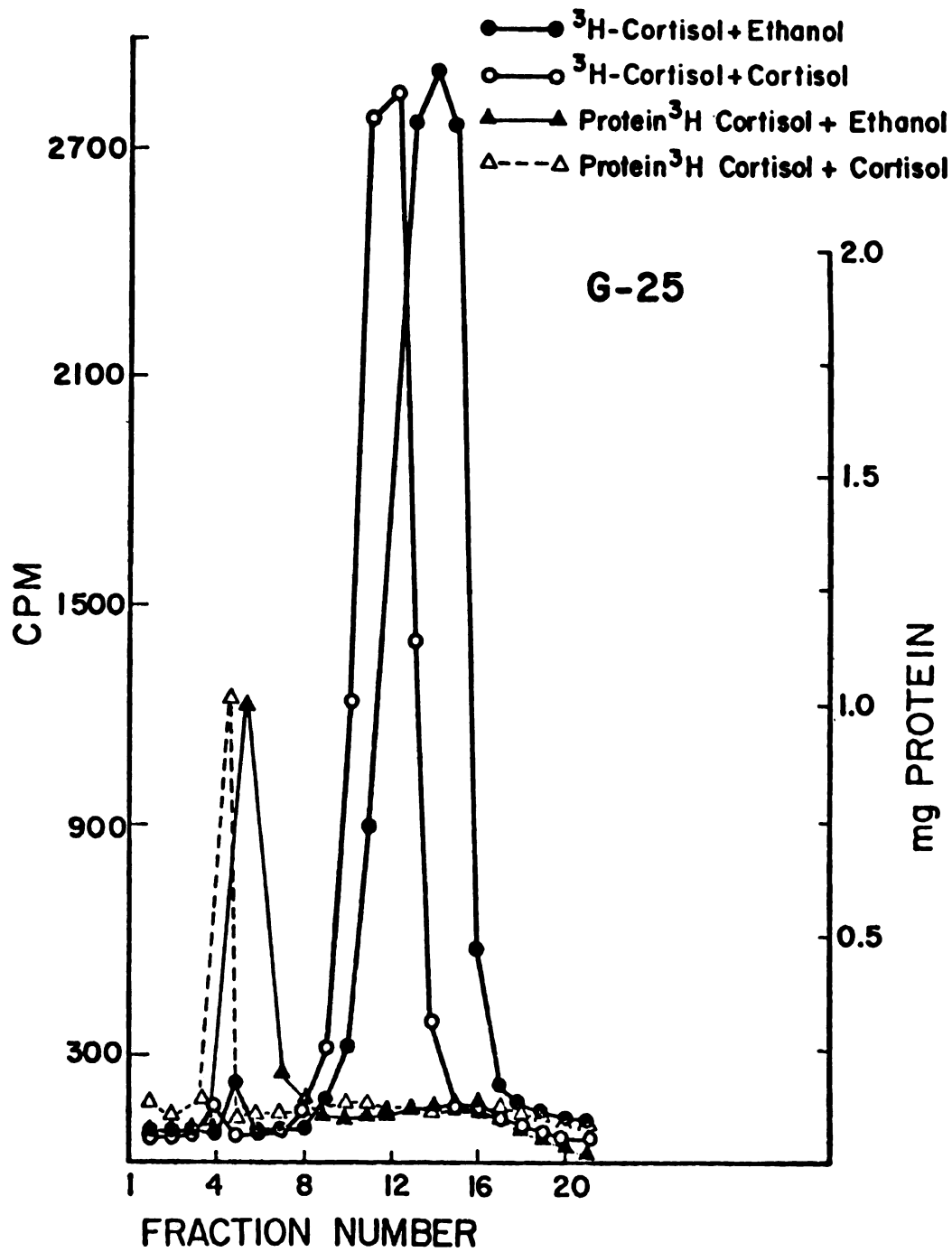
Figure 12

700 x g and 100,000 x g Precipitates

Unlabelled cortisol reduced the binding of ^{3}H-cortisol when directly incubated with 700 x g precipitates suspended in 199: MEM by 21% ($p < 0.05$), Tris-EDTA buffer by 19% ($p < 0.05$), 700 x g mammary tissue supernatants by 15% ($p < 0.05$) and bovine serum albumin by 17% (Table 17). A reduction in binding of ^{3}H-cortisol, in the presence of unlabelled cortisol, was not observed when 700 x g precipitates were directly incubated in whole bovine serum (Table 17).

Microsomal pellets (100,000 x g precipitates free of 700 and 15,000 x g cellular components) bound large amounts of ^{3}H-cortisol. However, this binding was not specific since unlabelled cortisol or dexamethasone consistently failed to reduce binding of ^{3}H-cortisol (Table 18).

Physiological Effects of Corticoids
on Mammary Tissue Slices
From Lactating Cows

Triamcinolone-Cortexelone Competition

When compared with ethyl alcohol controls, unlabelled triamcinolone, cortexelone, cortisol and dexamethasone added to mammary slices reduced the binding of ^{3}H-cortisol in 700 x g supernatant fractions by 32.1, 31.1, 34.9 and 37.7% respectively; and by 50.0, 44.4, 53.6 and 61.1%, respectively, in 700 x g precipitates (Table 19).

Table 17. Binding of ^{3}H-cortisol to cell-free isolates of 700 x g precipitates of mammary tissue slices from lactating cows.[a]

Incubation media	DPM/μg Total DNA[c]	
	^{3}H-cortisol + ethyl alcohol	^{3}H-cortisol + cortisol
199 MEM	1.4	1.1[b]
Tris-EDTA buffer	1.6	1.3[b]
Mammary 700 x g supernatant	1.3	1.1[b]
1% bovine serum albumin	1.2	1.0[b]
Bovine serum	0.5	0.5

[a] Concentrations of ^{3}H-cortisol and unlabelled cortisol were 1.6×10^{-9} M and 4×10^{-8} M in ethyl alcohol, respectively.

[b] Less than respective ethyl alcohol control ($p < 0.05$).

[c] Total DNA was measured in 700 x g precipitate after ethyl alcohol extraction of ^{3}H-corticoid and was used to adjust data.

Table 18. Binding of ^{3}H-cortisol to cell-free isolates of 100,000 x g precipitates (microsomes) of mammary tissue slices from lactating cows.

Hormone	CPM $\pm$ SE
^{3}H-cortisol[a] + ethyl alcohol	36,650 $\pm$ 202
^{3}H-cortisol + cortisol[b]	34,482 $\pm$ 121
^{3}H-cortisol + dexamethasone[b]	36,329 $\pm$ 210

[a] ^{3}H-cortisol concentration was 1.6 x 10^{-9} M in ethyl alcohol.

[b] Unlabelled cortisol and dexamethasone concentrations were 4 x 10^{-8} M in ethyl alcohol.

Table 19. Triamcinolone and cortexelone inhibition of ^{3}H-cortisol and ^{3}H-dexamethasone binding to 700 x g supernatant and precipitate fractions of mammary tissue slices from lactating cows.[a]

Unlabelled hormone	^{3}H-cortisol DPM/μg Total DNA[d]		^{3}H-dexamethasone DPM/μg Total DNA[d]	
	700 x g supernatant	700 x g precipitate	700 x g supernatant	700 x g precipitate
Triamcinolone	72[c]	0.9[c]	138[b]	3.0[c]
Cortexelone	73[c]	1.0[c]	140[b]	3.3[c]
Cortisol	69[c]	0.8[c]	136[b]	2.5[c]
Dexamethasone	66[c]	0.7[c]	129[b]	1.2[c]
Progesterone	100	1.6	148	4.0
17β-estradiol	105	1.9	156	3.9
Ethyl alcohol control	106	1.8	150	5.2

[a]Concentration of unlabelled steroids was 6.7×10^{-8} M in ethyl alcohol, while the concentration of ^{3}H-cortisol and ^{3}H-dexamethasone was 2.7×10^{-9} M in ethyl alcohol.

[b]Less than ethyl alcohol control ($p < 0.05$).

[c]Less than ethyl alcohol control ($p < 0.01$).

[d]Total DNA was measured in 700 x g precipitate after ethyl alcohol extraction of ^{3}H-corticoids and was used to adjust data in 700 x g supernatant and precipitate fractions.

However, progesterone and 17β-estradiol did not signifi-
cantly (p > 0.05) reduce binding of ^{3}H-cortisol in either
fraction (Table 19.

When ^{3}H-dexamethasone was used in place of ^{3}H-cortisol
and the various unlabelled hormones were added to mammary
slices as described above, triamcinolone, cortexelone,
cortisol and dexamethasone reduced the binding of ^{3}H-dexa-
methasone in 700 x g supernatants by 8.0, 6.7, 9.3 and 14.0%
respectively; and by 42.3, 36.5, 51.9 and 76.9% respectively,
in 700 x g precipitates (Table 19). Progesterone and 17β-
estradiol did not significantly (p > 0.05) effect ^{3}H-dexa-
methasone binding in 700 x g supernatant or precipitate
fractions.

C^{14}-Glucose Uptake

Unlabelled cortexelone, cortisol, triamcinolone and
dexamethasone inhibited C^{14}-glucose incorporation into
mammary tissue slices from lactating cows (Figure 13).
Inhibition of C^{14}-glucose incorporation was apparent with
all corticoids at hormone concentrations as low as 10^{-8} M
when compared with ethyl alcohol controls (Figure 13).
Progesterone served as a second control. No inhibition of
C^{14}-glucose was observed with progesterone. As the concen-
tration of corticoids increased, inhibition of C^{14}-glucose
uptake increased in a dose response relationship (Figure 13).
Inhibition of C^{14}-glucose incorporation was greatest at

Figure 13. Effects of various unlabelled corticoids on
C^{14}-glucose uptake into mammary tissue slices
from lactating cows at 37°C. The percent inhi-
bition of C^{14}-glucose uptake was calculated
from controls (100% uptake, 0% inhibition).

o———o cortexelone (11-deoxycortisol)

●———● cortisol

x———x triamcinolone

Δ———Δ dexamethasone

——— progesterone

▲———▲ ethyl alcohol controls
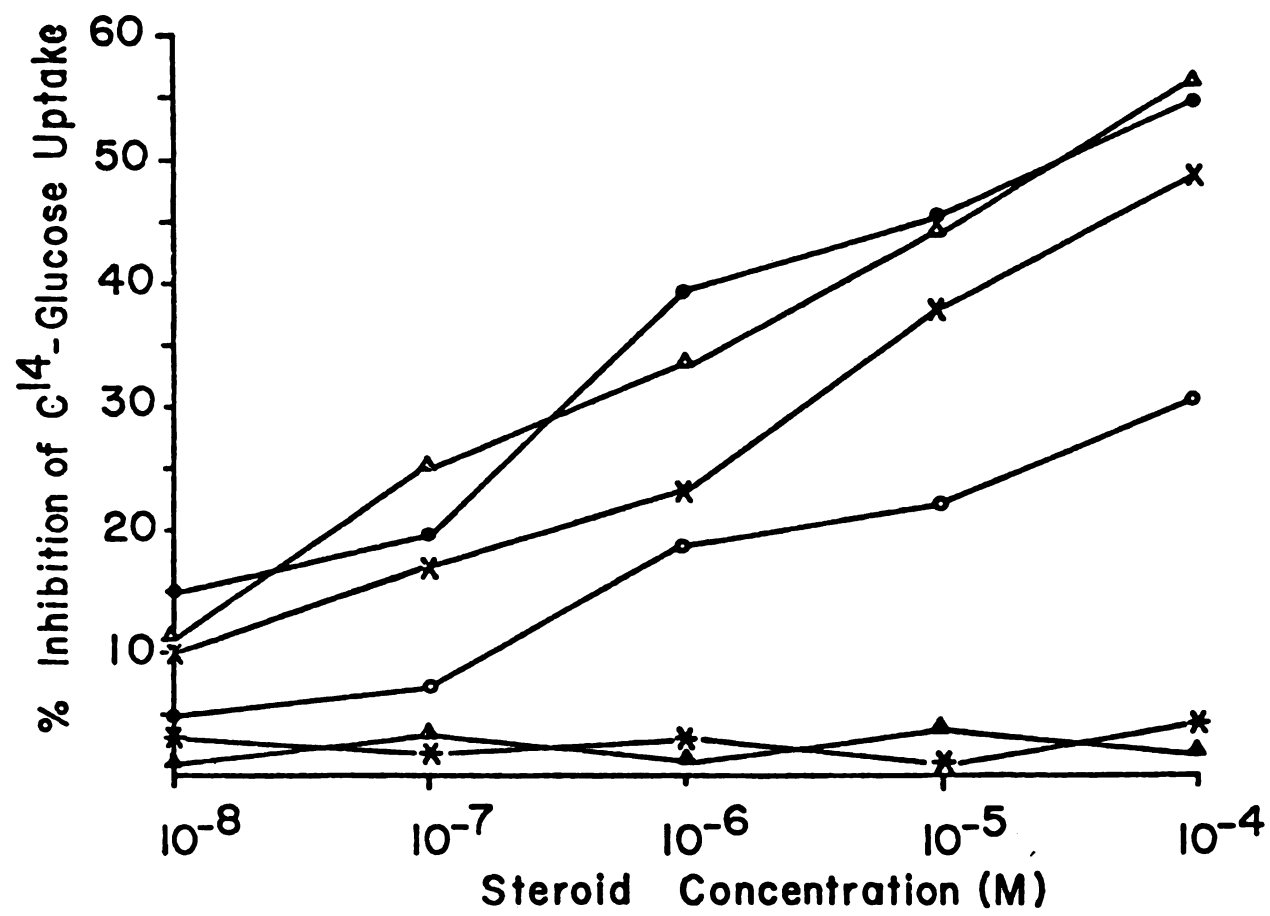
Figure 13

hormone concentrations of 10^{-4} M, when compared with ethyl

alcohol controls. At concentrations of 10^{-4} M, cortisol

and dexamethasone reduced the uptake of C^{14}-glucose into

mammary tissue by approximately 55% when compared with ethyl

alcohol controls. Triamcinolone and cortexelone reduced

glucose uptake 48 and 30%, respectively, at concentrations

of 10^{-4} M, when compared with ethyl alcohol controls

(Figure 13).

The inhibition of ^{3}H-cortisol and ^{3}H-dexamethasone

uptake by unlabelled corticoids served as a semi-quantitative

index of corticoid binding in 700 x g supernatant and pre-

cipitate fractions of mammary tissue slices. Product-moment

correlation analysis showed that corticoid binding in 700 x

g supernatant fractions of mammary tissue slices from

lactating cows was significantly ($p < 0.01$) correlated

($r = 0.91$, for ^{3}H-cortisol and C^{14}-glucose; $r = 0.93$, for

^{3}H-dexamethasone and C^{14}-glucose) with the inhibition of

C^{14}-glucose uptake.

In other experiments, 1 x 10^{-6} M cortisol significantly

($p < 0.05$) reduced the uptake of C^{14}-glucose into mammary

tissue 42%, while 1 x 10^{-4} M cortexelone significantly

($p < 0.05$) reduced C^{14}-glucose uptake by 30% (Table 20).

When cortisol and cortexelone were simultaneously added to

mammary tissue slices from lactating cows, glucose uptake

was reduced by only 4.0% (Table 20). Cortexelone, therefore,

interacted with cortisol to increase C^{14}-glucose uptake

into mammary tissue slices.

Table 20. C^{14}-glucose uptake into mammary tissue slices from lactating cows.

Treatment	CPM/μg Total DNA ± Std Error	$[\frac{c-e}{c} \times 100]^f$
Ethyl alcohol control	36.0 ± 0.25	%
Cortisol (1×10^{-6} M)	$20.8^a \pm 0.50$	42.
Cortexelone (1×10^{-4} M)	$25.1^a \pm 0.43$	30.
Cortisol (1×10^{-6} M) plus cortexelone (1×10^{-4} M)	$34.5^b \pm 0.36$	4.

[a] Less than control ($p < 0.05$).

[b] Greater than uptake seen for cortisol or cortexelone alone ($p < 0.05$) but not significantly different from control ($p > 0.05$).

$c = C^{14}$-glucose uptake of control without corticoid.

$e = C^{14}$-glucose uptake with corticoid.

[f] Total DNA was measured in 700 x g precipitates and was used to adjust data.

In Vivo Experiments

Mammary Uptake of Corticoids

Table 21 shows the external pudic artery and mammary vein concentration (ng/ml) of serum corticoids before and after milking. This table also illustrates arterial-venous concentration differences (ng/ml) for corticoids before and after milking. Serum corticoids, obtained from external pudic artery samples, averaged 6.72 ± 1.33 ng/ml (base line $\pm$ S.E. of mean) prior to the initiation of milking, at time zero (Table 21). At milking, arterial corticoids increased to 13.13 ± 1.14 ng/ml and continued to increase until twelve minutes after the initiation of milking to 17.34 ± 1.02 ng/ml. After this interval, corticoids decreased to a final level of 3.61 ± 0.95 ng/ml sixty minutes after the initiation of milking.

Serum corticoids, obtained from mammary vein samples, averaged 6.65 ± 1.19 ng/ml (base line) prior to the initiation of milking (Table 21). At milking, venous corticoids increased to 14.82 ± 1.40 ng/ml, dropped slightly at six minutes, then increased again at twelve minutes after the initiation of milking to 14.45 ± 1.11 ng/ml. After this interval, venous corticoids decreased to a final level of 3.55 ± 1.0 ng/ml sixty minutes after the initiation of milking.

Table 21. External pudic artery and mammary vein concentration of serum corticoids before and after milking.

Time in minutes prior to (-) and after (+) milking	-30	-15	-6	0[a]	+6	+12	+16	+20	+30	+60
				Total corticoids (ng/ml)						
Arterial	5.05	5.76	9.35	13.13	16.54	17.34	12.02	10.04	8.12	3.61
Venous	4.56	6.74	8.64	14.82	10.73	14.45	13.68	10.19	8.98	3.55
Arterial-Venous difference	+0.49	-0.98	+0.71	-1.69	+5.81[b]	+2.89	-1.66	-0.15	-0.86	+0.06

[a] Milking was initiated and continued for approximately 5 minutes.

[b] Significantly greater than zero (p < 0.10).

S.E. of any A-V difference = $\sqrt{MS_{Ed}/\text{\# observations per A-V difference mean}}$

Arterial-venous differences in corticoids were positive at 6 and 12 minutes after the initiation of milking. The mean A-V differences at these time intervals were 5.81 ng/ml $\pm$ 2.40 (mean A-V difference $\pm$ SE of mean) and 2.89 ng/ml $\pm$ 2.40, respectively (Table 21). These positive A-V differences in corticoids were not statistically significant from zero at the $p < 0.05$ level (Table 21).

DISCUSSION

Unlabelled cortisol, cortexelone, triamcinolone, and
dexamethasone significantly reduced the binding of tritiated
cortisol and dexamethasone in 700 x g supernatant and precip-
itate fractions subsequently isolated from mammary tissue
slices of lactating cows (Tables 1, 2 and 19). Unlabelled
progesterone, testosterone and 17β-estradiol did not signifi-
cantly reduce corticoid binding to mammary tissue from
lactating cows (Tables 2, 7 and 19). These results provided
qualitative evidence that mammary tissue slices from lactat-
ing cows were capable of specifically binding cortisol and
dexamethasone in 700 x g supernatant and precipitate frac-
tions. Unlabelled corticoids also reduced uptake of
labelled triamcinolone acetonide and dexamethasone in mam-
mary tissues from lactating rats (Gardner and Witliff,
1973a), mice (Shyamala, 1973a) and voles (Turnell et al.,
1974b). However, other noncorticoid hormones reduced uptake
of various corticoids in these systems. For example,
progesterone reduced binding of various corticoids in rat
(Gardner and Witliff, 1973a), mouse (Shyamala, 1973a) and
vole (Turnell et al., 1974b) mammary tissues and progester-
one and 17α hydroxyprogesterone reduced binding of cortisol

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in cultured bovine mammary cells (Tucker et al., 1971).

Furthermore, Gardner and Witliff (1973a) reduced tritiated

triamcinolone binding in mammary tissue from lactating cows

with high doses (5 x 10^{-6} M) of unlabelled 17β-estradiol,

and Turnell et al. (1974b) reduced tritiated triamcinolone

binding in mammary tissue from lactating voles with high

doses (5 x 10^{-6} M) of 17β-estradiol and 0.5 to 5 x 10^{-6} M

testosterone. We conclude that the bovine mammary tissue

slice system may be more specific for binding corticoids

than those reported by other workers.

Unlabelled cortisol and dexamethasone were also capable

of reducing tritiated cortisol and dexamethasone binding in

mammary tissue slices from virgin heifers, one-month prepar-

tum and dry cows (nonpregnant, nonlactating) (Tables 5-8).

Only subtle differences were apparent, however, between the

results previously described for mammary tissue slices from

lactating cows. For example, unlabelled dexamethasone

reduced tritiated dexamethasone binding in 700 x g precipi-

tate fractions of virgin mammary tissue, while unlabelled

cortisol did not (Table 5). Unlabelled cortisol did not

reduce tritiated dexamethasone binding in 700 x g super-

natant fractions from dry cows (Table 8). Furthermore, the

total uptake of tritiated corticoids, in the absence of

unlabelled corticoids, was greater in mammary tissue slices

from lactating cows when compared with mammary tissue from

cattle in other physiological states (Table 7). The greater
uptake of corticoids observed in mammary tissue slices from
lactating cows was believed to be associated with lactation-
al events occurring within mammary cells. The total uptake
of tritiated dexamethasone was greater than tritiated corti-
sol in tissue slices from one month prepartum and lactating
cows when compared with virgin heifers and dry cows (Tables
5-8). The enhanced uptake seen for dexamethasone, when com-
pared with cortisol, might have been attributable to lacta-
tional events or to the greater biological activity of
dexamethasone (Westphal, 1971 and Yoshitaka et al., 1966).

Gardner and Witliff (1973a) demonstrated that unlabelled
corticoids reduced triamcinolone acetonide binding in mammary
tissue from pregnant rats. However, these workers failed to
reduce tritiated triamcinolone binding in virgin rat mammary
tissue with the addition of unlabelled corticoids. They
suggested that this response was due to the paucity of re-
ceptor molecules for triamcinolone in virgin rat mammary
tissue.

Competitive hormone binding experiments are not quanti-
tative. Scatchard plot analysis, however, allows one to
calculate several constants which are directly related to
the kinetics of hormone-receptor interactions. For example,
association constants (Ka) and dissociation constants (Kd =
$\frac{1}{Ka}$) for hormone receptor complexes, can easily be calculated

from Scatchard plots (Scatchard, 1949). The total number
of hormone molecules bound per cell can also be determined.
This in turn gives an estimate of the number of hormone
receptor sites per cell, if one assumes that the receptor
site binds one molecule of hormone per molecule of receptor.

Scatchard analyses of mammary tissue slices from lac-
tating cows revealed a single high affinity binding component
($Kd \sim 10^{-10}$ M) for cortisol and dexamethasone. This constant
was in close agreement with those found in mammary tissues
of other species, which ranged from 6×10^{-9} to 10^{-7} M
(Gardner and Witliff, 1973a; Shyamala, 1973a and Turnell
et al., 1974b). The 1299 to 1957 (adjusted) molecules of
corticoids bound with high affinity to fresh mammary tissue
slices from lactating cows, were less than the 7500 mole-
cules of cortisol bound with high affinity to bovine mammary
cells cultured in vitro (Tucker et al., 1971) and less than
the number of molecules of corticoid bound to mammary tissue
from other species (Shyamala, 1973a; Gardner and Witliff,
1973a and Turnell et al., 1974b). The low number of corti-
coid molecules bound in mammary tissue slices from lactating
cows may have been attributable to stress, induced at
slaughter, in cattle used in this study. This stress may
cause elevated serum corticoids which would then bind to
specific corticoid receptor sites. The number of receptor
sites detectable by Scatchard assay would therefore be
underestimated.

Mammary tissue from lactating cows bound significantly more total molecules of dexamethasone than cortisol. The greater number of apparent binding sites for dexamethasone may have been a result of the synthetic corticoid's greater biological activity (Westphal, 1971 and Yoshitaka et al., 1966).

A single homogeneous population of specific corticoid receptor molecules was observed in these studies and has been described for a number of other mammary tissue systems (Gardner and Witliff, 1973a; Shyamala, 1973a and Turnell et al., 1974b). However, the fact that only one high affinity component for corticoid binding was observed for cortisol and dexamethasone in our system was contrary to results observed in cultured bovine mammary cells where two high affinity components bound cortisol (Tucker et al., 1971). The discrepancies between these two systems may have been a result of cellular dedifferentiation in cultured cells, with a resultant change in receptor site affinity or synthesis of a mixed population of receptors.

In addition to the high affinity binding component, another component that had a low affinity, but larger capacity for corticoid was apparent in mammary tissue slices from lactating cows (Figure 1A and 1B). This component was thought to be analogous to the nonspecific component found in cultured bovine mammary cells (Tucker et al., 1971).

The component was present in all preparations studied and could not be eliminated by washing the tissue. Attempts were made to eliminate this component from 700 x g supernatant fractions by charcoal and florisil treatment, but this resulted in absorption of bound cortisol from specific receptor sites. Mathematical transformation of Scatchard data was therefore necessary to subtract nonspecific binding in tissue fractions which might have been associated with the specific high affinity binding component (Appendix D). This correction allowed for a more precise estimation of receptor-hormone dissociation constants and numbers of corticoid molecules bound per mammary cell.

Scatchard plots carried out on tissue slices from lactating cows at 37°C and 4°C indicated that, similar to many steroid hormone binding systems (Sandberg et al., 1966) the mechanism for corticoid binding was temperature dependent. Reducing the incubation temperature did not markedly alter the affinity of mammary tissue for cortisol, but the total number of molecules bound with high affinity was reduced by approximately 82% (Table 3).

Fresh mammary tissue slices from lactating cows did not metabolize cortisol prior to its becoming bound in the 700 x g supernatant (Figure 3). This was unlike the results found for liver cells (Baxter and Tomkins, 1971a) and mammary cells cultured in vitro (Tucker et al., 1971) where a

substantial portion of cortisol recovered from the cytosol was in a form other than authentic cortisol. The absence of bound metabolites of cortisol in cytosol fractions of fresh mammary tissue from lactating cows was analogous to that observed for rat thymus (Munck and Wira, 1971) and mouse mammary tissue (Shyamala, 1973a). Further experiments are needed in order to determine if cortisol is metabolized prior to becoming bound in 700 x g mammary nuclear fractions.

Experiments designed to determine if the uptake of corticoids into mammary tissue slices from lactating cows was dependent on a facilitated active transport system were carried out using 10^{-5} mM Ouabain (cardiac glycoside). This concentration of Ouabain was chosen, since it blocks the Na^{+}/K^{+} pump in cardiac and smooth muscles (Repke, 1963). Ouabain had no effect on uptake of cortisol. Therefore, it was assumed that corticoid uptake into mammary slices from lactating cows was either passive or that uptake could possibly be blocked by other transport inhibitors or other concentrations of Ouabain.

Scatchard analyses of cortisol and dexamethasone binding in virgin heifers, one-month prepartum and dry cows revealed a two component corticoid binding system which showed some similarities with those previously described for mammary tissue from lactating cows. For example, one component had high affinity for corticoid and was saturable.

The other component had low affinity for cortisol and proved unsaturable.

In general, no specific trends among reproductive states were observed for Kd's of corticoids bound to either 700 x g supernatant or precipitate fractions, even though statistical analyses revealed significant differences (Tables 9 and 11). The number of corticoid molecules bound in 700 x g supernatant and precipitate fractions also varied significantly among reproductive states (Tables 10 and 12). But, specific trends were present when the number of corticoid molecules bound per mammary cell were compared among reproductive states. For example, mammary slices from virgin heifers and dry cows bound fewer molecules of cortisol when compared with prepartum and lactating cows (Table 10). There was no significant difference between the number of molecules of cortisol bound in tissue fractions of virgin heifers and dry cows (Table 10). Furthermore, lactating cows bound more molecules of dexamethasone than virgin heifers and dry cows (Table 12). As a general rule, mammary tissue slices from all cattle bound approximately 2.0 times more molecules of dexamethasone than cortisol. This might be explained by the well-known greater biological activity of dexamethasone (Westphal, 1971 and Yoshitaka et al., 1966).

Interpretation of competitive hormone binding experiments and Scatchard analyses of corticoid binding to whole

mammary tissue slices is somewhat restricted because the tissue slices represent a mixed population of cell types. The experimenter can not be certain of the binding in a particular cell type. Results of binding experiments would, therefore, vary from one experiment to the next depending upon the number of specific cell types present which are capable of specifically binding corticoid. However, characterization of corticoid receptors in mammary tissue from lactating rats (Gardner and Witliff, 1973a), mice (Shyamala, 1973a) and voles (Turnell et al., 1974) has recently been reported in the literature. These receptors were characterized as proteins, since binding was altered by proteolytic enzymes and mercurials. Evidence presented in this dissertation suggested that the corticoid receptors in mammary tissue from lactating cattle were proteins, because trypsin (Table 15) and p-chloromercuribenzoate (Table 16) significantly reduced specific cortisol binding in 700 x g supernatant fractions of mammary tissue slices from lactating cows.

Results of hormone binding experiments carried out on cattle in various reproductive states may be brought into focus if it is assumed that corticoid binding is specific for mammary alveolar epithelial cells (Tucker et al., 1971) or proteins closely associated with these cells (Gardner and Witliff, 1973a; Shyamala, 1973a and Turnell et al.,

1974b) and that the physiological function of corticoids, and other mammotropic hormones, is involved with growth and maintenance of metabolically active alveolar cells.

The mammary gland of virgin heifers consists primarily of ducts and connective tissue. The degree of development is primarily dependent upon the number of estrous cycles the animal has after puberty. In general, estrogen causes proliferation of ductular epithelial tissue, whereas progesterone causes proliferation of alveolar epithelium. The few alveolar epithelial cells present in the virgin mammary gland are not representative of the metabolically active milk secreting epithelial cells of the pregnant and lactating animal and have no dependence on lactogenic hormones. The paucity of receptor sites for corticoid (Tables 10 and 12) in mammary tissue from virgin heifers could be attributable to the lack of alveolar epithelial cells. Gardner and Witliff (1973a) have also reported a low number of corticoid receptor sites in mammary cytosol fractions from virgin rats when compared with pregnant or lactating rats.

During pregnancy, the mammary gland, with its rudimentary ductular structure is transformed into fully secreting tissue. Ovarian, pituitary, cortical and pancreatic hormones (mammotropic hormones) are believed to act synergistically throughout this transformation to stimulate the production of metabolically active alveolar cells.

Cellular differentiation progresses throughout pregnancy and remains dependent upon corticoids and other mammotropic hormones. Lumina of terminal ducts and alveoli are swollen with proteins and other secretory products. At this stage of mammary development, the gland is similar to the lactating gland, with the exception that "milk" is not being produced. The large numbers of metabolically active alveolar cells present in one-month prepartum mammary tissue could conceivably contribute to the greater number of corticoid receptor sites when compared with virgin and dry cows (Tables 10 and 12).

Shortly before parturition, there is an increase in serum concentrations of corticoids and other lactogenic hormones, as well as a decrease in progesterone. These responses are thought to affect the initiation of lactation. The fully lactating mammary gland represents the summit of cellular differentiation. Alveolar cells are completely efficient metabolic factories for the production of casein, milk, fat, β-lactoglobulin, lactose and other milk components. It is conceivable that the rise in corticoids and other lactogenic hormones seen shortly before parturition, may stimulate alveolar cells to produce milk components and specific species of protein hormone receptors. The abundance of metabolically active (protein synthesizing) alveolar cells could contribute to the greater number of corticoid

receptor sites when compared with virgin heifers, one-month

prepartum and dry cows (Table 10 and 12). Gardner and

Witliff (1973a) reported more specific corticoid binding in

mammary glands of lactating rats as opposed to the glands

of virgin and pregnant rats. Furthermore, Emery (1969)

showed a sixfold increase in corticosterone binding in lac-

tating rats when compared with animals in their sixteenth

day of pregnancy.

At the end of lactation, regression of the mammary

gland takes place. This response is believed to be a result

of changes in lactogenic and mammotropic hormone concentra-

tions which may be rate-limiting to milk secretion and mam-

mary development. Alveoli lose their structural integrity

and become engorged with lipid droplets and protein granules.

The overall anatomy of mammary glands from dry cows (non-

pregnant, nonlactating) resembles that for virgin heifers.

There are very few, if any, functional epithelial cells and

the gland is primarily composed of connective tissue and

ductular epithelium. Some nonsecretory alveolar cells

associated with terminal ducts remain intact. The reduction

in metabolic activity and associated regression of alveolar

epithelial tissue caused by the reduction of rate limiting

lactogenic and mammotropic hormones could account for the

paucity of receptor sites seen in mammary tissue slices from

dry cows (Tables 10 and 12). This could also account for

the close agreement between the number of binding sites observed in dry cows and virgin heifers.

Several attempts were made in this study to relate specific corticoid uptake and binding to physiological responses occurring within mammary tissue. Cameron (1973) and Turnell et al. (1974a) demonstrated that glucocorticoids inhibited uptake of C^{14}-glucose into mouse mammary tissue slices and rat thymocytes, respectively. In this dissertation, cortisol, cortexelone, triamcinolone and dexamethasone were effective in inhibiting C^{14}-glucose incorporation into mammary tissue slices from lactating cows (Figure 12). The concentrations of corticoids necessary to inhibit glucose incorporation were slightly greater than those used to achieve saturation of the high affinity binding component in Scatchard analyses. The high positive correlation between corticoid binding and inhibition of C^{14}-glucose incorporation by corticoids suggested that these responses were closely related.

Further confirmation of these data was provided in experiments using cortexelone and cortisol. Turnell et al. (1974a) and Munck and Wira (1971) showed that cortexelone does not have the biological activity that most glucocorticoids have on glucose uptake into thymus tissue, but that it is an avid competitor for tritiated-triamcinolone and tritiated-cortisol binding. When cortexelone plus

triamcinolone or cortisol were simultaneously added to thymus preparations, cortexelone acted as an antiglucocorticoid and reduced the ability of corticoids to inhibit glucose incorporation. These workers proposed that cortexelone occupied specific binding sites which were normally occupied by more biologically active corticoids. Cortexelone was thought to affect the stereochemistry of the hormone receptor complex, so that it no longer exerted its normal physiological action on the cell. When cortisol and cortexelone were simultaneously added to mammary slices from lactating cows in this study, glucose uptake was increased. This suggested that cortexelone interacted with cortisol to increase glucose incorporation into mammary tissue slices. Since cortexelone reduced corticoid binding in mammary tissue slices from lactating cows and had reduced biological activity with regard to inhibiting C^{14}-glucose incorporation, when compared with cortisol, it was further suggested that corticoid binding to specific receptor molecules in mammary tissue slices from lactating cows was definitively correlated with glucose uptake.

In vivo experiments designed to measure the arterial-venous concentration differences of corticoids across the mammary gland suggested that corticoids were taken up by the mammary gland shortly after the onset of milking. The mean A-V differences at 6 and 12 minutes after the start of

milking (5.81 ng/ml and 2.89 ng/ml) were comparable to A-V

differences in corticoids observed by Patterson and Linzell

(1974) in lactating cows. Whether or not the uptake was

specific to the mammary gland and represented utilization

of corticoids for milk secretion or was merely nonspecific

and a result of increased arterial corticoid concentrations

remains to be elucidated. The rise in arterial and venous

corticoids noted at 30 and 15 minutes before milking (Table

21) was believed to be a result of exteroceptive stimuli

induced by the presence of the milker or milking unit.

Similar results have been reported by Smith et al. (1972)

who showed that the milking stimulus per se and exterocep-

tive stimuli caused increased serum corticoids in cows.

Hormone binding experiments, described in this disser-

tation, suggested that the specific corticoid binding

component of mammary tissue from lactating cows was not a

result of tissue contamination with the corticoid binding

globulin (CBG) of blood. For example, CBG has a higher

affinity for progesterone at 37°C than for corticoids

(Rosenthal et al., 1969; Sandberg et al., 1966; and Seal

and Doe, 1966) but, tritiated cortisol or tritiated dexa-

methasone binding to mammary tissue could not be competi-

tively inhibited with unlabelled progesterone at 37°C.

Furthermore, dexamethasone is only a moderate to poor com-

petitive inhibitor of cortisol binding to CBG (Pizarro,

1969; Peets et al., 1969 and Baxter and Tomkins, 1971a).
But, dexamethasone proved to be an excellent competitive
inhibitor of cortisol binding in the bovine mammary slice
system. Also, the binding affinity of CBG to cortisol is
reduced by decreasing the incubation temperature (Sandberg
et al., 1966). As described earlier, reducing the tempera-
ture from 37° to 4°C did not effect the affinity of cortisol
binding to mammary tissue slices from lactating cows, but
did reduce the total number of molecules specifically bound
with high affinity. In experiments where the binding of
tritiated cortisol, dexamethasone and progesterone was
examined in sera, sera bound more cortisol and progesterone
at 37°C when compared with dexamethasone. This gave further
confirmation that the corticoid receptor found in mammary
tissue was unique.

Additional attempts were made to distinguish corticoid
binding in mammary tissue from that of blood using several
physicochemical techniques. The results of these attempts
confirmed the hormone binding experiments previously
described. Namely, the corticoid binding component of mam-
mary tissue was unique and not a contaminate from blood
(CBG). For example, gel filtration chromatography and
sucrose gradient analyses of mammary supernatant fractions
suggested that the protein(s) binding cortisol was a macro-
molecule with a molecular weight of approximately 2.5×10^5

to 3×10^6 (Figures 6 and 7); whereas, the major protein which bound cortisol in blood had an approximate molecular weight of 6 to 8×10^4 (Figures 6 and 7). Disc gel electrophoresis and DEAE cellulose chromatography confirmed that the corticoid binding protein(s) of mammary tissue had different electrochemical characteristics than serum binding components.

The physicochemical techniques employed to characterize the mammary corticoid receptor in this study did not completely resolve the biochemical nature of the binding protein. Future attempts are necessary to determine the structure of this macromolecule. The size of the cytosol binding protein suggested that it may be composed of subunits. These subunits may be nucleic acid(s) carbohydrate(s) or other proteins which do not participate in the binding reaction.

Intact tissue was not a necessary determinant for specific binding of cortisol to mammary tissue in isolated 700 x g supernatant or precipitate (containing microsomes and other cellular debris) fractions, because unlabelled cortisol reduced tritiated cortisol binding in these fractions (Figure 12, Table 17). In contrast, isolated 100,000 x g precipitate (microsomes) devoid of other cellular debris did not specifically bind cortisol (Table 18).

Evidence presented in this dissertation suggested that mammary tissue slices possessed protein(s) receptor molecules which were capable of specifically binding corticoids. The fact that mammary tissue slices from lactating cows bound more molecules of corticoids than tissue from cattle in other reproductive states and that binding and uptake of corticoids appeared related to events associated with milk secretion, may form the basis for further studies to determine the specific role which corticoids play in lactation. Based on the data reported in these studies, one could speculate that specific corticoid binding is involved in the growth and maintenance of metabolically active alveolar epithelial cells.

SUMMARY

Evidence presented in this dissertation suggested that bovine mammary tissue slices possessed specific receptor molecules for corticoids. Specificity for corticoids was based on the observations of hormone binding experiments and Scatchard analyses.

Unlabelled cortisol, cortexelone, triamchinolone and dexamethasone reduced binding of tritiated cortisol and dexamethasone in mammary tissue slices from lactating cows. Unlabelled progesterone, testosterone and 17β-estradiol had no effect on corticoid binding in fresh mammary tissue slices from lactating cows. Scatchard analysis revealed two components which bound cortisol and dexamethasone in mammary slices from lactating cows. One component had high affinity ($Kd \sim 10^{-10}$ M) for both corticoids and was saturated at low hormone concentration. The other component had low affinity for both corticoids and was practically unsaturable. It is believed that the high affinity component represented specifically bound corticoid. The unsaturable component was thought to represent nonspecifically bound hormone. Mammary tissue from lactating cows bound approximately 1263 and 1855

total molecules of cortisol and dexamethasone, respectively, per mammary cell.

Attempts were made to compare corticoid binding in mammary tissue slices from lactating cows with binding in mammary tissue slices from cattle in other physiological states. These physiological states included virgin heifers, 1-month prepartum and dry (nonpregnant, nonlactating) cows. Results of competitive hormone binding experiments, carried out using tritiated cortisol and dexamethasone and the other unlabelled steroids mentioned above, were similar to those described for mammary tissue slices of lactating cows. Scatchard analyses of corticoid binding in mammary tissue slices from virgin heifers, 1-month prepartum and dry cows showed similarities to those for mammary tissue slices from lactating cows. For example, a two component Scatchard plot was resolved for each physiological state. Dissociation constants (Kd) for cortisol and dexamethasone ranged from 0.3 to 20 x 10^{-10} M. Mammary slices from lactating cows, bound more molecules of corticoid per mammary cell when compared to the other physiological states examined. Virgin heifers and dry cows bound the fewest number of corticoid molecules per mammary cell. Mammary tissue slices from all physiological states examined bound more molecules of dexamethasone than cortisol. Thin-layer chromatography of specifically bound tritiated cortisol, in 700 x g supernatants

of mammary tissue slices from lactating cows, indicated that the majority of bound radioactivity was authentic cortisol.

Gel filtration chromatography experiments showed that the specific corticoid receptor in 700 x g 15,000 x g and 100,000 x g supernatants and 700 x g precipitate fractions of mammary tissue slices from lactating cows was a macro-molecular protein(s). This protein(s) had an approximate molecular weight of from 2.5×10^5 to 3×10^6. Binding of tritiated cortisol to this protein(s) was reduced by tryp-sin and mercurials. Further biochemical analyses of the corticoid binding protein(s) in mammary tissue from lactat-ing cows, using the techniques of disc gel electrophoresis, DEAE cellulose chromatography and gel filtration chromatog-raphy, showed that the protein(s) was unique to mammary tissue and not a contaminate from blood (CBG).

Cell-free preparations of 700 x g supernatants and precipitate fractions specifically bound cortisol. Cell-free preparations of 100,000 x g precipitates bound large quantities of tritiated cortisol, however, this binding was not specific since unlabelled corticoids failed to reduce binding of tritiated cortisol.

Glucose uptake into mammary tissue slices from lactat-ing cows was reduced by various unlabelled corticoids. The ability of cortisol to inhibit glucose incorporation was

reduced by the addition of cortexelone (11-deoxycortisol). Cortexelone reduced tritiated cortisol binding in fresh lactating tissue slices and proved to have an antiglucorticoid action on glucose uptake. Correlation analyses suggested that specific corticoid binding in mammary tissue slices from lactating cows was correlated with glucose incorporation into the mammary cell. Furthermore, corticoids were taken up from the blood by the mammary gland shortly after initiation of the milking response.

In summary, data presented in this dissertation suggested that mammary tissue slices possessed protein(s) receptor molecules which were capable of specifically binding corticoids. The fact that mammary tissue slices from lactating cows bound more molecules of corticoids than tissue from cattle in other physiological states and that binding and uptake of corticoids appeared related to events associated with milk secretion, may form the basis for further studies to determine the specific role which corticoids play in lactation.

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APPENDICES

APPENDIX A

SCINTILLATION FLUID

For 700 x g Supernatants
and Aqueous Solutions

>Beckman Biosolve-3 (600 ml)
>Toluene (3 liters)
>2,5-diphenyloxazole (15 gm)
>1,4-bis[2-(5-phenyloxazoyl)]benzene (0.9 gm)

For 700 x g Precipitates

>Toluene (3 liters)
>2,5-diphenyloxazole (15 gm)
>1,4-bis[2-(5-phenyloxazoyl)]benzene (0.9 gm)

APPENDIX B

QUENCH CORRECTION

Percent Efficiency

    Standards

        Count tritium standards to determine actual
        counts per minute (cpm).

$$\text{Percent Efficiency} = \frac{\text{cpm (standard}}{\text{disintegrations per minute}} \atop \text{(dpm) of standard}$$

External Standard Ratio (ESR)

    Standards

    1. Count tritium standards with external
       standardization. Print out will give:

Sample No.	Time	Channel A	Channel B	
4123	1000	247	290	Sample
4123	40	116	117	Sample
4123	40	273	157	Sample + Ext. Standard 133 B$_a$

    2. for Channel A = 273 - 116 = 57

       for Channel B = 157 - 117 = 40

$$\text{ESR:} \quad A/B = \frac{\text{Channel A (57)}}{\text{Channel B (40)}}$$

    3. plot standard curve:

       X axis = ESR A/B (standards)
       Y axis = Percent Efficiency (standards)

       Linear regression of standard curve was
       carried out on an Olivette programable
       desk computer.

Quench Correction and Determination of
DPM for Unknowns

1. Follow steps 1 and 2, knowing the A/B for unknown,
   read percent efficiency from standard curve.

2. $\dfrac{100\%}{\text{Percent Efficiency (unknown)}}$ = Factor Q

3. DPM for unknown = Multiply Factor Q by 10 minute
   unknown sample count for Channel.

APPENDIX C

DNA ASSAY

Reagents:

    Ether (anhydrous)
    5% TCA (trichloroacetic acid)
    95% ethyl alcohol saturated with sodium acetate
        $(C_2H_3N_aO_2)$
    1 N sodium hydroxide (KOH)
    6 N hydrochloric acid (HCl)
    10% PCA (perchloric acid)
    5% PCA

Procedure:

    Part I

    1. Sample contains 700 x g precipitate (nuclear frac-
       tion) plus 3 ml of ethyl alcohol (ETOH)

    2. Centrifuge 15 min at 18,000 RPM, pour off ETOH,
       add 3 ml ether.

    3. Centrifuge 15 min at 18,000 RPM, decant ether super-
       natant and discard.

    4. Evaporate pellet, 20-30 min under air, add 5 ml
       5% TCA (ice cold), centrifuge at 18,000 RPM for
       15 min. Carefully decant supernate, discard.

    5. To pellet add 5 ml 5% TCA, centrifuge at 18,000 RPM
       for 15 min. Carefully decant supernate, discard.

    6. Add 5.0 ml of 95% ETOH sodium acetate, vortex,
       centrifuge at 18,000 RPM for 15 min.

    7. Discard supernate, save pellet, pellet is used
       immediately or can be frozen.

Part II

1. To pellet obtained from Part I, step number 7, add 4.0 ml of 1N KOH, vortex, incubate at 37°C for 15 hrs.

Part III

1. Add 4 ml ice cold 6N HCl to pellet in Part II, step 1. Then add 5 ml of ice cold 10% PCA.

2. Centrifuge at 18,000 RPM for 15 minutes, decant supernate (RNA) and discard.

3. Wash pellet with 5 ml of ice cold 5% PCA, vortex, centrifuge at 18,000 RPM for 15 min, decant and discard supernate.

4. To pellet, add 5 ml of 5% PCA, vortex and incubate at 70°C for 15 min, centrifuge at 18,000 RPM for 15 min, decant off supernate into 25 ml volumetric flask.

5. To pellet, wash with 5 ml additional 5% PCA, vortex and centrifuge at 18,000 RPM for 15 min, add supernate to volumetric flask.

6. To pellet, repeat step 5, volumetric contains 15 ml of 5% PCA extract. Bring volume up to 25 ml with additional 5% PCA.

7. Read percent transmission or spectrophometer set at 268°A, convert to optical density (O.D.) from table.

8. µg DNA = O.D. x 47 µg/ml x 25 mls.

SCATCHARD PLOT TRANSFORMATION CORRECTION
FOR NONSPECIFIC BINDING

Scatchard plots were corrected for nonspecific binding by the following mathematical and statistical transformations:
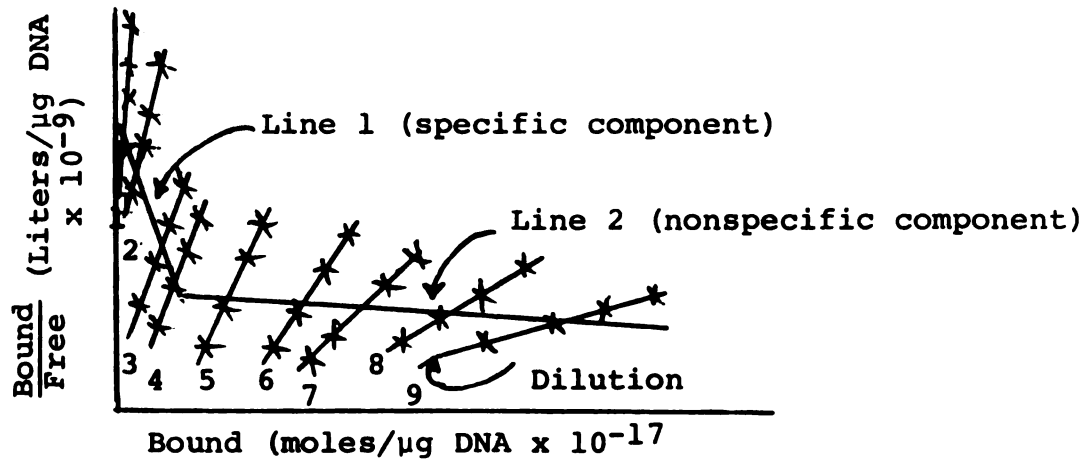

Figure A1. Scatchard plot unadjusted for nonspecific binding.

Two Component Curve Analysis

1. Two separate least squares analyses were carried out for points on lines one and two of Figure A1. Each point (x's) represents a mean value from quadruplicate samples from a particular animal, obtained in four separate experiments, at one of a total of nine hormone dilutions.

2. Linear regression analysis was used to calculate the equation for line 2 (Figure A1) for each physiological state Scatchard plot. The regression equation is represented by equation 1.

$$\hat{Y} = b_o + b_1 X \text{ (equation 1)}$$

$$b_o = y \text{ intercept for line 2}$$

$$b_1 = \text{slope for line 2}$$

$$Y = \frac{bound}{free}$$

$$X = bound$$

Transformation to produce "best fitting" linear regression line representing high affinity-specific corticoid binding component.

a. Substitute bound values (X) corresponding to Y experimental points on line 1 into linear regression equation 1.

b. Obtain $\hat{Y}$ for each point on all four dilutions of line 1.

c. Y (corrected) = Y (experimental) - $\hat{Y}$

d. Plot Y (corrected) values against bound (experimental) values for dilutions of line 1 (Figure A1).

e. Least squares analysis was performed on the means for each dilution to give a single line representing high affinity binding (line 3, Figure A2, on the following page).
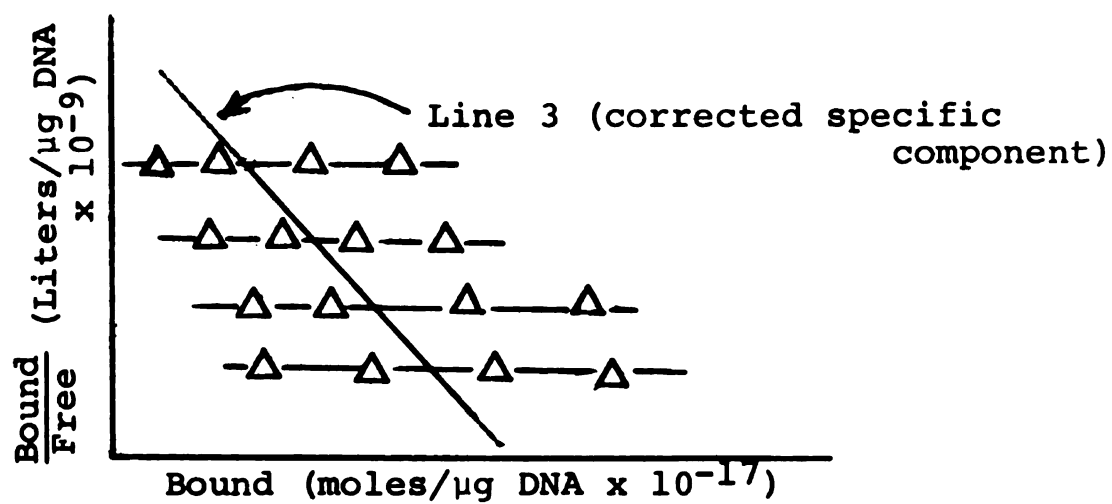
Figure A2. Scatchard plot adjusted for nonspecific binding.

Δ represents $\dfrac{bound}{free}$ (corrected) vs bound (experimental) values for each animal at each of four hormone dilutions.

APPENDIX E

LOWRY METHOD FOR PROTEIN DETERMINATION

Reagents:

A. Lowry A

   1. Sodium carbonate (anhydrous)   60.0 g
   2. Sodium hydroxide (pellets)   12.0 g
   3. Sodium or potassium tartrate   0.6 g
   4. Distilled H_2O to make   3,000.0 ml

B. Lowry B

   1. Copper sulfate solution   0.5 g%
      ($CuSO_4 \cdot 5H_2O$)

C. Lowry C (prepared fresh daily)

   1. Lowry A   50 parts
   2 Lowry B   1 part

D. Phenol reagent according to Folin Ciocalteu

   1. Phenol Reagent-concentrate   1 part
      (Central Scientific Co.)

   2. Distilled H_2O 1 part

E. Protein Standard 8.0 g% (Dade Reagents Inc.,
   Miami, Fla. Lot No. PRS-406)

   1. Dilute with 100 ml distilled H_2O to give 800
      µg/ml

Concentrations of protein standards used for determina-
tion of standard curve: 0, 20, 40, 60, 80 and 160 µg/ml.

Procedure:

1. 1 ml of protein solution (standard or unknown) added to 5 ml of Lowry C.

2. Incubate 20 min at room temperature.

3. 0.5 ml phenol reagent jetted in for rapid mixing.

4. Incubate 1/2 hr at room temperature (20-22°C), mix occasionally.

5. Read at 600 mμ.

APPENDIX F

DISC GEL ELECTROPHORESIS[a]

Buffers:

   A. 6.0 gm TRIS
      0.8 ml concentrated HC   100 ml distilled H_2O

   B. 0.75 gm TRIS
      0.40 ml Concentrated HCl   100 ml distilled H_2O

   C. 0.6 gm TRIS   2.0 liters distilled H_2O
      3.0 gm glycine

Running Gel:

   1.4 gm cyanogum -41 for 7% gel
   20 ml buffer A
   0.02 ml TEMED
   0.02 gm Ammonium Persulfate

Sample Gel:

   4.0 gm acrylamide
   5.0 gm sucrose
      100 ml Buffer B
   0.1 gm Ammonium Persulfate, 0.1 ml TEMED

Spacer Gel:

   0.4 gm Cyanogum -41 in 10 ml buffer B
   0.1 ml TEMED
   0.01 gm Ammonium Persulfate

----

   [a]Samples were electrophoresed 4-5 hrs, 2 ma/disc at
      4°C.

APPENDIX G

COMPETITIVE PROTEIN BINDING ASSAY
FOR SERUM CORTICOIDS

Duplicate aliquots of serum (0.2 mls) are placed in 16 x 100 mm test tubes. In order to account for procedural losses, approximately 3,000 dpm of 1, 2, 6, 7 ^{3}H-cortisol (specific activity 91 ci/mmole) is added to a third aliquot from a series of unknowns (10 to 20 within each assay).

Samples are vortexed vigorously for 1 minute with 2.0 ml nanograde 2, 2, 4 trimethyl pentane to remove progesterone. After extraction, samples are stored at -20°C for 1 hour. The solvent layer is decanted and discarded before the serum is thawed. Serum is allowed to warm to room temperature. After the samples reach room temperature, 2.0 ml of reagent grade methylene chloride is added in order to extract the corticoids. Samples are vortexed vigorously for 1 minute. After separation of solvent and serum, the lower methylene chloride layer, containing corticoid is carefully aspirated off with a pasteur pipette and saved in a 12 x 75 mm test tube for assay.

Cortisol (Sigma Chemical Co.) is pipetted from a stock solution of 10 ng/ml in ethyl alcohol (100%) for use as standards. Three sets of standards (0.0, 0.1, 0.25, 0.5,

1.0, 1.5, 2.0, 2.5, 5.0, long) are included in each assay.

Solvent in standard and unknown tubes is evaporated under air, and 1.00 ml of 1.25% dog plasma with approximately 40,000 dpm/ml 1, 2, 6, 7 ^{3}H-cortisol, is added to each tube.[a] Assay tubes are then stored at 4°C for 12 to 18 hours.

After incubation at 4°C, assay tubes are placed in an ice bath and allowed to equilibrate for at least 15 minutes. In order to separate bound from free corticoids, 0.5 ml of 1% dextran T70 (Pharmacia, Uppsula, Sweden) and 0.5% carbon decolarizing, neutral norit (Fisher Scientific Co.) in glass distilled water is added to each tube. Samples are then rapidly mixed and allowed to incubate in an ice bath for 5 minutes, then centrifuged at 2,000 x g for 15 minutes at 4°C. A 0.5 ml aliquot of the supernatant is diluted with 5.0 ml of liquid scintillation cocktail (3a 70B, Research Products International Corp., Elk Grove Village, Ill.) for quantification of radioactivity. Standard sera with high and low corticoids are assayed with each set of unknown serum samples.

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[a]Dog plasma (Colorado Serum Co.) is diluted to 2.5% in 500 ml distilled water and mixed with 60 g Florisil (80 mesh; Matheson, Coleman and Bell) for 3 hours to eliminate endogenous steroids. The suspension is then centrifuged at 2,800 rpm for 15 minutes. The supernatant fluid volume is doubled with glass distilled water to give 1.25% plasma. One, 2, 6, 7 ^{3}H-cortisol is added to the 1.25% plasma to give approximately 40,000 dpm/ml and can be stored at 4°C for up to one month.

STATISTICAL ANALYSIS OF SCATCHARD
PLOT SLOPES AND X INTERCEPTS

Multiple comparison of Scatchard plot slopes (Kd's)

1. $\hat{Y} = b_o + b_1 X$ (linear regression equation for line 3, Figure A2)

   $X_o = (\dfrac{-b_o}{b_1})$ = x intercept; b_o = y intercept; b_1 = slope

2. Determine SSx_i and Sp^2 (pooled error variance)* for lines.

3. Test statistic: for $(b_i - b_i')$ Scheffe, minimum significant difference between

$$b_i \; \} \; b_i' \; \pm \sqrt{(t-1)f_{0.05, \; t-1, \; V_E}[S_p^2 \; (\frac{1}{SSx_i} + \frac{1}{SSx_i})]}**$$

   t = number treatments being compared

   V_E = degrees of freedom for error

   * pooled error variance, Sp^2 =

$$\sqrt{\frac{SS_{E_1} + SS_{E_2}}{df_1 + df_2}}$$

   ** $V (b_1 - b_i') = [Sp^2(\frac{1}{SSx_i} + \frac{1}{SSx_i})]$

Multiple comparison of Scatchard plot x - intercepts (molecules of corticoid bound in mammary cell)

   Z = molecules of corticoid bound in mammary cell =

$$\frac{-c_1}{c_2} \; (\frac{b_o}{b_i})$$

b_o, from line 3 Figure A2.

b_1, from line 3 Figure A2.

1. $V(Z) = (\frac{c_1^2}{c_2^2})\ V\ [(b_o/b_1)]$

$\cong (\frac{c_1^2}{c_2^2})\ [b_o^2\ V\ (b_1) + b_1^2\ V\ (b_o) - 2\ b_o b_1\ CoV$

$(b_o b_i)]/b_1^4$

$V(b_1) = (MSE/SSx),\ b_o = \bar{y} - b_1\ \bar{x}$

$CoV\ [(\bar{y} - b_1\ \bar{x}),\ b_1] = -\ \bar{x}\ V\ (b_1)$

$V(b_o) = MSE\ [(\frac{1}{n}) + \bar{x}^2/SSx]$

approximate standard error of Z = S.E. = $\sqrt{V(Z)}$

2. Test differences in x-intercepts (molecules bound) using the following Scheffé confidence interval:

$(Z_1 - Z_2) \pm \sqrt{(t-1)\ f_{\alpha,\ t-1,\ Ve(p)}\ **[V(Z_1) + V(Z_2)]}$

$\alpha = 0.05$

$\alpha = 0.01$

t = number treatments being compared

** pooled degrees of freedom for error