ABSTRACT

PURIFICATION OF A STEROID BINDING PROTEIN IN MAMMARY GLAND

By

Donald H. Steyert

Adrenal glucocorticoids are important in several tissues for a number of functions. Among those functions is their apparent essentiality in the lactation process at both the general metabolic and mammary tissue levels. At the tissue level, binding of a hormone is generally thought to indicate a biological function for that hormone in that tissue. This study was to determine whether such binding occurred in mammary tissue and if it did to partially purify and study the binding material.

Binding from 2.5×10^{-8} M corticosterone was observed in centrifugally produced nuclear, mitochondrial and microsomal fractions of mammary tissue at 41.1%, 27.4%, and 31.1% of the total particulate label in those three respective fractions. The remaining soluble, or

cytoplasmic, fraction also had considerable binding activity, revealed during Sephadex filtration.

That soluble binding fraction was then partially purified by ammonium sulfate precipitation, calcium phosphate gel and DEAE-cellulose adsorption and Sephadex filtration. The binding protein was initially precipitated from solution by 60-80% saturated ammonium sulfate and that fraction, stable to frozen storage, became the focus of further purification and binding studies.

Calcium phosphate gel or Sephadex G-100 filtration provided little or no further purification. However, DEAE-cellulose eluted with phosphate buffers constituting ionic and pH gradients did provide purity beyond that in the ammonium sulfate fraction. Absolute degree of purification could not be determined due to apparent high levels of low-affinity, nonspecific binding in the starting material. Although DEAE-cellulose fractionation provided the highest purity of binding protein there were still several bands observable by polyacrylamide gel electrophoresis.

Competition and binding studies are facilitated by separation of bound and unbound hormone. Adsorption

of unbound steroid to Florisil, charcoal and dextran- and polyvinylpyrrolidone-coated charcoals on Millipore filters proved to be unsatisfactory. Dialysis of samples against charcoal in a buffer suspension proved unsatisfactory due to dissociation of bound steroid during dialysis. Equilibrium dialysis does not separate unbound from bound hormone but does produce data for equilibrium conditions and as such was used for both competition and binding constant studies.

Competition for corticosterone binding sites was observed for both hydrocortisone and progesterone but progesterone competition appeared less complete. Neither cholesterol nor 17β-estradiol competed with corticosterone.

Two sets of binding constant data were obtained. One set was produced with ammonium sulfate fractions from two mammary tissues dialyzed 12 hours against four corticosterone concentrations. The second set of data was from the same fractions of two other mammary tissues, one of them duplicated, but they were dialyzed 24 hours against 12 corticosterone concentrations. The first data gave high affinity binding constants between 0.96 and 4 X 10^{-9}M from Scatchard and double reciprocal plots. The second

data gave, by linear regression calculations on a Scatchard plot, a high affinity binding constant of 3.2×10^{-11}M. The discrepancy between the two may result from closer spacing of the low concentration points or from the increased dialysis time.

Relationships between blood corticosteroid-binding globulin and mammary glucocorticoid-binding protein may exist. Furthermore, relationships between those two and levels of progesterone and glucocorticoid may relate to lactogenesis. During lactogenesis, near parturition, blood levels of progesterone decrease while free glucocorticoid levels increase. Since the mammary binding protein strongly binds both of these steroids the decreasing progesterone concentration may permit increased glucocorticoid binding which may thus involve this mammary protein in the initiation of lactation.

VITA

Donald Harrison Steyert was born December 8, 1940, in Brooklyn, New York. His parents, Harrison and Sybil, and younger sister, Jo-Ann, lived fourteen years in Massapequa Park, New York, before moving to Orwell, Vermont. After attending two high schools in New York and two in Vermont he was graduated from Hudson Falls Central High School, New York, in 1958. He attended the University of Vermont from 1958 to 1962 and received a BS degree in Animal Science. He received the MS degree from Michigan State University, Department of Dairy, under the supervision of Dr. R. S. Emery in 1965. The title of his thesis was "Glucose Assimilation by Rumen Microbes and Effect of Penicillin." He was then employed by the Michigan State University Department of Veterinary Surgery and Medicine for work on a canine leukemia project for one year. He will now receive a Ph.D. degree from the Dairy Science Department, Michigan State University, in 1971. Since January of 1971 he has been an assistant professor in the Dairy Science Department at the University of Illinois on temporary appointment for one year. He is a member of the American Dairy Science Association and Alpha Zeta.

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INTRODUCTION

Adrenal glucocorticoid hormones have great influence upon the body. The research conducted and reported herein was designed to add one more piece of information concerning the relationship of glucocorticoids to one specific tissue, the mammary gland.

The importance of glucocorticoids in mammary development and in the initiation and maintenance of lactation is concluded from a review of the pertinent literature. Binding of a hormone in a tissue is an indication for its biological role in that tissue. Binding of estrogenic hormones and glucocorticoids has been shown in a number of tissues. This study was performed to show the existence of macromolecular binding of glucocorticoid hormone in mammary tissue.

REVIEW OF LITERATURE

Adrenal Glands and Their Secretions

The adrenal glands, which lie near the kidneys, are each composed of two distinct portions; each secreting a different type of hormone. The inner portion, or adrenal medulla, secretes epinephrine and norepinephrine while the other portion, the adrenal cortex, secretes steroid hormones called corticosteroids. The adrenal cortex also secretes small quantities of androgenic and estrogenic hormones (1). Corticosteroids are further classified as mineralocorticoids and glucocorticoids.

The mineralocorticoids are primarily responsible for maintaining electrolyte blance while the glucocorticoids, of which hydrocortisone (cortisol, or 17α-hydroxycorticosterone) and corticosterone (4-pregnene-11β, 21-diol-3,20-dione) are the major molecules, are important in carbohydrate, protein, and lipid metabolism.

The adrenal cortex synthesizes the glucocorticoids from acetyl CoA or cholesterol formed elsewhere in the

body. Secretion of these hormones is under the control of adrenocorticotropin (ACTH) secreted from the anterior pituitary gland (1).

Ninety-five percent of the glucocorticoid activity in the human results from hydrocortisone secretion while a small amount comes from corticosterone (2) and a minute amount from cortisone (1). Corticosterone is the major circulating glucocorticoid in the rat (3) and mouse (4,5); however, the bovine has both corticosterone and hydrocortisone but blood concentration of both is only one-tenth that of the rat (6,7,8).

The best known affect of glucocorticoids on metabolism is their stimulation of gluconeogenesis in the liver. By increasing gluconeogenesis and possibly by decreasing cellular glucose utilization, glucocorticoids increase the blood level of glucose. The gluconeogenic effect of glucocorticoids increases the catabolism of cellular protein and decreases the rate of protein synthesis in all tissues except the liver where both liver protein content and output of plasma proteins are increased (1).

Glucocorticoids also promote lipid mobilization. They increase both the rate of incorporation into adipose

tissue and the rate of removal from adipose tissue by accelerating many of the different processes involved in lipid metabolism (1).

In Vivo Effects of Glucocorticoids

Liver

The tissue showing the most widespread response to glucocorticoids is the liver which would be expected considering the importance of both glucocorticoids and liver in gluconeogenesis and lipid and carbohydrate metabolism. The liver usually responds to glucocorticoids or to increased dietary protein by increasing its enzyme activity.

Induction of numerous liver enzymes has been studied by administration of both exogenous glucocorticoid and increased dietary protein. Szepesi and Freedland (9) cite numerous examples of rat liver enzymes affected by both glucocorticoid treatment and increased dietary protein. Among them are tryptophan pyrrolase, tyrosine-α-ketoglutarate transaminase, serine dehydrase, glutamic-pyruvic transaminase, glutamic-oxalacetic transaminase,

glucose-6-phosphatase and certain enzymes of the urea cycle, amino acid catabolism and gluconeogenesis. Hormonal effects on enzyme turnover rates were also studied. The rates of synthesis of two of the enzymes studied, glucose-6-phosphatase and glutamic-pyruvic transaminase, were increased by hydrocortisone without changes in their rates of degradation. The rate of synthesis of another enzyme, serine dehydrase, was increased by both hydrocortisone and increased dietary protein while only hydrocortisone increased its half-life (9).

Mammary Gland

The development of the mammary gland is a stepwise process starting with duct development followed by formation of the secretory tissue and finally secretory activity. The hormonal requirements for duct development were studied by Lyons et al. (10) using hypophysectomized-ovariectomized-adrenalectomized rats. They found that estrogen plus growth hormone gave some duct growth; however, a glucocorticoid administered along with the estrogen and growth hormone yielded full duct growth.

The secretory tissue of the mammary gland, consisting primarily of lobules of alveoli, develops after duct development. Some lobule-alveolar development was produced in rats by estrogen plus progesterone plus growth hormone; however, the addition of a glucocorticoid to these hormones resulted in full development and milk secretion (10).

Lobule-alveolar development histologically similar to midpregnancy development in the intact mouse has been produced in hypophysectomized-ovariectomized-adrenalectomized virgin mice by <u>in vivo</u> hormone treatment. Growth hormone plus estradiol and progesterone produced the best development. Lactogenesis was induced in the mice by a fifteen-day treatment with estradiol, progesterone and growth hormone plus five daily injections of hydrocortisone acetate and growth hormone. The histological appearance of the glands in these rats was very similar to that of the normal lactating gland (11). Similar results have also been observed in the goat (12) indicating that growth hormone and prolactin act in conjunction with ovarian and adrenal cortical hormones to produce full alveolar development.

Hormonal initiation of lactation has also been studied. ACTH or adrenal corticoids will initiate lactation in all species in which they have been tried providing well developed mammary glands are present in the animal prior to treatment. ACTH injections initiated lactation in pseudopregnant rabbits (13) and corticoid treatment initiated lactation in pregnant rats (14,15) and rabbits (15) and pseudopregnant rabbits (16). Predef (9-fluoroprednisilone), a synthetic adrenocortical steroid, was used by Tucker and Meites (17) to initiate lactation in early, mid-, or late pregnancy heifers. They suggested that "adrenal cortical steroids are limiting factors for initiation of lactation in pregnant heifers, as in laboratory animals." There is some evidence that plasma concentration of free adrenal glucocorticoids increases at the time of parturition (7).

Much evidence exists suggesting glucocorticoids are also very important in the maintenance of lactation. The ACTH content of the anterior pituitary was found to decrease 68% between the twentieth and thirty-sixth day of lactation in rats (18). This period is also associated with greatly decreased milk production.

In vivo glucocorticoid treatments have been used to study sustentation of lactation. Lactation in adrenalectomized rats could not be supported by mineralocorticoids alone but added glucocorticoids acted synergistically to maintain lactation (19,20). Cortisone acetate significantly increased lactation during the first 18 days postpartum (21) and hydrocortisone acetate prevented the drastic reduction in litter weight gain normally observed between 24 and 32 days of lactation in rats (22). Hydrocortisone acetate was injected with and without exogenous prolactin in order to study effects on mammary cell RNA and DNA. Injections from day 16 to 32, without prolactin, maintained mammary cell numbers, measured as gland DNA content; however, metabolic activity per cell (RNA/DNA) decreased (22). Injections of both hydrocortisone acetate and prolactin had the inverse effect. These hormones reduced the DNA content but increased the RNA/DNA ratio. Thatcher (23) also showed that Predef injections started on day 24 maintained litter weight gains, DNA levels and gave a high RNA/DNA ratio to day 32. It would be possible that the decreased lactational performance found during the latter portions of corticoid treatments could be due to nutrients becoming limiting and increased

caloric intake has improved lactation performance during Predef treatment (24).

ACTH, contrary to its effects in rats, decreased lactational performance in cows (8,25). Injection of ACTH caused a sharp drop in milk yield from which maximum recovery took at least three days (8). There was also a rapid, marked increase in plasma 17-hydroxy corticosteroid levels which fell sharply in a few hours and returned to normal within 24 to 48 hours.

Activities of a number of enzymes have been studied with respect to the effect of onset of lactation upon them. Activities of 6-phosphogluconate dehydrogenase, phosphoglucomutase and phosphofructokinase in the rabbit all increase between one day prepartum and one day postpartum and these increases follow the DNA increase seen over the same period (26). Acetyl CoA carboxylase follows the same pattern but citrate cleavage enzyme continues to increase in activity to at least 10 days of lactation, the time when DNA levels decrease. Hartmann (26) reported the observed changes in the rabbit were consistent with the pattern seen in the rat, mouse, and guinea pig; however, the changes were not as marked as those seen in the rat (27) and guinea pig (28). The marked increases in

the activity of enzymes seen with the onset of lactation in those two species was not observed in the cow where the enzymatic potential for milk synthesis was present at least 14 days prepartum (28).

A number of mammary enzymes are affected by _in vivo_ treatments of prolactin and hydrocortisone. These two hormones in combination significantly increased glucose-6-phosphate dehydrogenase and fatty acid synthetase in hypophysectomized-rat mammary glands (29,30, 31). Levels of 6-phosphogluconate dehydrogenase and phosphoglucomutase were also increased by combined prolactin and hydrocortisone, while malate dehydrogenase and fructose 1,6-diphosphate aldolase were increased by either prolactin or hydrocortisone (29,30). Citrate cleavage enzyme and UDPG-pyrophosphorylase were both significantly increased by hydrocortisone and further increased by the addition of prolactin (31).

With the exception of glucose-6-phosphate dehydrogenase, most of the enzymes mentioned above increased as DNA content increased thereby reflecting gland growth and cell proliferation (29). Glucose-6-phosphate dehydrogenase activity per mg DNA was increased by hydrocortisone plus prolactin but not by either one alone. The

activity of another enzyme, aspartate amino transferase, increased slightly per mg DNA by either hydrocortisone or prolactin (29).

In Vitro Effects of Glucocorticoids

Mammary Gland

Numerous studies relating lactogenesis to specific hormones have been performed *in vitro* where experiments are often more convenient than *in vivo* and the tissue is removed from influences other than those being studied. Much of the *in vitro* work has used mouse mammary cultures to study histological changes following various hormone treatments. Glands removed from 14 day-pregnant mice show advanced lobule-alveolar development, but culturing this tissue in hormone-free medium resulted in extensive degradation of the parenchymal cells. Neither hydrocortisone, growth hormone, nor prolactin alone maintained this tissue; however, a combination of hydrocortisone and prolactin was effective in maintaining viability and the histological integrity of secretory tissue (32).

Insulin introduced into hormone-free culture medium improved the in vitro survival of 10-12 and 14-18 day-pregnant mouse mammary explants but did not maintain them as well as in combination with hydrocortisone (33, 34). Hydrocortisone alone would not maintain the normal histological appearance of these explants and some histological differences were noted between tissue in insulin-hydrocortisone vs the prolactin-hydrocortisone medium (33) used previously (32). The hydrocortisone requirement for maintenance of the mammary explant was also found to increase with the degree of differentiation (34).

Hormonal treatments have also been used to induce mammary secretion. The minimum hormone combination required for initiation of secretion from early-prelactating (10-12 day pregnant) mouse mammary was either prolactin or growth hormone plus insulin and hydrocortisone (34). The insulin-hydrocortisone-prolactin treatment produced differentiated alveolar cells which were not merely modified duct cells but arose only as the result of stem cell division in the presence of the three hormones (35).

Hydrocortisone, corticosterone, and aldosterone were all effective for induction and maintenance of normal

secretory histological appearance in mouse mammary explants (36). Using aldosterone as the model adrenal corticoid histological appearance was maintained with a minimum of 1 and 5 μg per ml of corticoid and insulin, respectively (37). The hormones at those levels also allowed prolactin and growth hormone to initiate secretion (37).

Steroid structural requirements for mammary gland differentiation have been determined. For intermediate activity at 3×10^{-7} M concentration the 4-pregnene steroid nucleus must have 1) a 20-keto group, and 2) an 11 β-ol or 21-ol. The requirement for the 3-keto group was not determined. Hydrocortisone, corticosterone, and aldosterone all meet the requirements for intermediate mammary gland differentiation. For increased activity at 3×10^{-7} M the 4-pregnene nucleus requires the 20-keto with either a 17α-hydroxyl or 18-aldehyde and either an 11 β-ol or 11-one (38). Hydrocortisone and aldosterone both meet those latter requirements. Deoxycorticosterone, found inactive at levels of 3×10^{-6} M and 1.4×10^{-5} M (36), meets the requirements for intermediate differentiation (38). Perhaps the relationships among the groups present on the nucleus is more important than was realized

in determining the requirements. Deoxycorticosterone has the 21-hydroxyl group required for intermediate activity and the 11-one required for a high degree of differentiation. However, the presence of the 11-one without the accompanying 17α-hydroxyl or 18-aldehyde may be enough to make it inactive. Corticosterone lacks the requirements for high activity although some in vitro work done with high concentrations of corticosterone showed it equivalent to hydrocortisone (36). There is some evidence that much higher levels of corticosterone than hydrocortisone were required in vivo for histological response (see 36).

Histological development of the mammary gland has been correlated with increased nucleolar size, increased RNA synthesis and initiation of casein synthesis (39). A number of studies related hormonal effects to both histological appearance and the onset of casein synthesis.

Virgin-mouse and pregnant-mouse mammary glands were treated with insulin, hydrocortisone, and prolactin to study incorporation of Pi^{32} into casein-like phosphoproteins. Virgin mouse mammary tissue showed little incorporation relative to pregnant mouse mammary (40). Proliferation of the duct system and more extensive alveolar development are probably required for

susceptibility to the triple hormone treatment (40). The pregnant mouse mammary was developed enough for the three hormones to synergistically elicit "casein" synthesis (40, 41) but none of the hormones alone or in pairs elicited that response (40). The histological development of the tissue was closely related to the elicitation of "casein" and both had the same triple hormone requirement for maximum response (35,40,42). Those phosphoproteins showing a stimulated synthesis were identical to the phosphoprotein present in mouse milk (43).

The rate of total casein synthesis was augmented three to sixfold by the triple hormone treatment (43). Insulin alone was not able to maintain either the characteristic pattern among the major casein components or the total capacity to make casein; however, the addition of hydrocortisone to the insulin treatment occasionally maintained the pattern but did not sustain total casein synthesis. Insulin plus prolactin gave some small stimulation of total casein synthesis but could not maintain the initial pattern. The triple hormone treatment produced a casein pattern like that found in the milk from 10-day lactating mouse tissue (43). Phosphoproteins other than

casein were not stimulated by insulin-hydrocortisone-prolactin treatment therefore the effect of the hormones must be very specific and not merely be to increase total protein synthesis or total cell numbers (39).

Further work elucidated the sequence in which the hormones exerted their effects. Insulin is primarily responsible for the DNA synthesis observed during cell proliferation in vitro (35,42) and the cytoplasmic non-milk protein synthesis believed to reflect that proliferation (44). In the absence of exogenous insulin immature (3-week old) mouse mammary, in contrast to adult tissue, will undergo DNA synthesis and mitosis (45). Such proliferation, however, did not lead to functionally differentiated cells (45). Insulin and hydrocortisone both had to be present during proliferation in order to elicit increased casein synthesis (42,45). The casein synthesis was induced in the absence of lobule-alveolar development; however, the normal alveolar development usually associated with initiation of casein synthesis could be induced in the immature mouse mammary gland after priming the animal with estradiol, progesterone and growth hormone. Then alveolar structures could be developed in vitro by insulin, hydrocortisone and prolactin (45).

The hormone-dependant casein synthesis was directly proportional to the rate of DNA synthesis, a reflection of proliferation occurring during that period (35). The DNA synthesis resulting from insulin treatment, and subsequent events in the cell cycle, were necessary for hormone dependent mammary differentiation (35,42).

Early reports indicated that cells which proliferated under the influence of insulin did not make casein unless hydrocortisone and prolactin were present during the proliferative period (42), but later work showed only hydrocortisone, and not prolactin, to be necessary during proliferation (46). In the more recent work tissue which had proliferated in insulin or insulin-prolactin medium showed no increase in casein synthesis upon post-proliferative insulin-hydrocortisone-prolactin treatment. Removal of hydrocortisone from the post-proliferative incubation still allowed augmented casein synthesis, indicating no post-mitotic effect for that steroid, but there was concern as to whether residual hydrocortisone from the insulin-hydrocortisone incubation could be expressing itself (46).

Insulin apparently also has a post-mitotic effect in mammary cultures (46). Cultures allowed to proliferate

in insulin-hydrocortisone then switched hydrocortisone-prolactin medium showed no augmentation of casein synthesis, thereby indicating insulin had to be present throughout in order to observe augmented casein synthesis.

Insulin's post-mitotic effect has been attributed to maintenance of tissue RNA levels (47,48). RNA synthesis has been measured by adenine incorporation into RNA of mouse mammary tissue cultured without insulin. After 12 hours the rate of synthesis decreased almost 27% and total RNA levels also fell. RNA synthesis in the presence of insulin gave higher initial incorporation which was maintained during the 12-hour incubation (47,48,49) and tissue RNA was nearly unaltered (47). However the insulin effect did not last for 48 hours of culture (48). Addition of corticosterone and prolactin to the insulin-culture-medium produced increased adenine incorporation into RNA starting at 6 hours and finally doubled the initial value with a lesser increase observed in insulin-hydrocortisone medium (48).

Prolactin has a post-mitotic action during casein induction as evidenced by the inability of colchicine to block its effect (46,50). Prolactin post-mitotically induced casein synthesis in mammary cells differentiated

by hydrocortisone. Lactalbumin and β-lactoglobulin synthesis was also augmented (50) and may reflect a further role for prolactin during initiation of lactation because the ratio of α-lactalbumin and β-lactoglobulin has been shown to increase as the lactational state approached although their combined amount increased proportionately to casein's increase (44). Most reports concerning hormone requirements for induction of casein synthesis show prolactin to be necessary but there is some evidence that growth hormone can substitute for prolactin (34).

Closely related to prolactin's effect on induction of milk protein synthesis is its effect on RNA synthesis (41). Prolactin-stimulated RNA synthesis was required to convert hydrocortisone-differentiated cells into secretory cells. The prolactin was also able to induce RNA synthesis in cells which had not been exposed to hydrocortisone but they were unable to make secretory proteins (51).

The best defined roles of insulin, hydrocortisone and prolactin are those just discussed but one further effect of hydrocortisone should be noted. In rat liver, either corticosterone or estradiol caused <u>in vitro</u> formation of rough endoplasmic reticulum from smooth

endoplasmic reticulum and polysomes (52). A similar study of the subcellular appearance of mouse mammary alveolar epithelial cells, also showed hydrocortisone able to induce formation of rough endoplasmic reticulum which was necessary for subsequent synthesis of secretory protein (53,54). In the mammary cells the hydrocortisone was used in conjunction with insulin, but insulin alone elicited little response (53). The formation of the rough endoplasmic reticulum provided an indication that hydrocortisone may have a post-mitotic effect because the cells containing rough membranes were formed whether hydrocortisone was added with insulin prior to daughter cell formation, or was added with insulin after proliferation (54).

Other Tissues

Glucocorticoid-induced synthesis of specific proteins in liver hepatoma has also been studied. As mentioned previously, hydrocortisone induces numerous enzymes in the liver, two of which are tyrosine-α-ketoglutarate transaminase and tryptophan pyrrolase. Tyrosine transaminase was studied in a tissue culture cell line

established from rats with ascites hepatomas (55). The hepatoma cells were found to be rapidly and substantially induced to produce increased tyrosine transaminase in response to a number of glucocorticoid hormones. The increased transaminase levels were due to increased synthesis and not decreased degradation (56) and there were glucocorticoid receptors detected having properties consistent with the increased transaminase synthesis (57).

The induction of tyrosine transaminase was found to be due to steroid antagonism of a labile post-transcriptional repressor of tyrosine transaminase (58, 59). Transcription of the tyrosine transaminase gene was repressed by a steroid-insensitive factor during the G2, mitosis and early G1 phases of the cell cycle. In late G1 phase both mRNA for tyrosine transaminase and the steroid sensitive repressor appear. During this later G1 phase the presence of steroid allows translation of the tyrosine transaminase mRNA. Transport of this RNA to the site of translation may be enhanced or its degradation may be decreased by the steroid antagonism of the repressor (59). The mention of transport to the site of action should bring to mind the evidence, mentioned in a previous section, concerning the ability of

glucocorticoids to mediate the formation of the rough endoplasmic reticulum required for secretory protein synthesis. The mRNA which directs the protein synthesis must be transported from the nucleus to the endoplasmic reticulum and glucocorticoid enhancement of the transport could promote formation of rough endoplasmic reticulum.

One other tissue in which glucocorticoid induction of an enzyme has been shown is human skin fibroblasts where prednisolone induced alkaline phosphatase (60).

Histones

As early as 1943 there were proposals (61) that histones (low molecular weight basic nuclear proteins) act as gene regulators or modifiers. Busch (62) notes that although the basis for such a concept is weaker now there are a number of authors who have reported evidence supporting histone suppression of nuclear function and one of the most convincing works showed the suppression of RNA synthesis in the nucleus was not merely due to histone inhibition of nuclear ATP synthesis (63).

Recent studies of histone synthesis in mammary gland have provided some indications of possible

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relationships between histones and initiation of lactation. Using the mammary culture system of Stockdale and Topper (42), Hohmann and Cole (64) compared histone synthesis in mammary glands cultured in insulin and insulin-hydrocortisone-prolactin. As proliferation of the glands occurred, total synthesis of lysine-rich histones paralleled DNA synthesis and chromatographic fractions of the lysine-rich histones showed definite effects due to hormone treatment. Synthesis of one (Fraction 1) of the five major fractions was reduced by exposure to the triple hormone treatment for 16-24 hours while exposure for 24-32 hours produced a much larger reduction of Fraction 1 but increased synthesis of Fractions 4 and 5. The authors concluded that such dramatic effects preceding the maximum induction of casein synthesis were related to the eventual synthesis of casein.

Histone synthesis in the mammary gland has also been studied <u>in vivo</u> (65). With the onset of lactation the lysine-rich and arginine-rich histones were synthesized at 2-3 times higher rates than the slightly lysine-rich fraction. The specific activities of all five lysine-rich fractions increased with the onset of lactation but Fractions 1 and 3 increased the least. After

six days lactation the specific activities decreased and Fraction 1 decreased more than any of the others. These *in vivo* changes in rates and patterns of histone synthesis from late pregnancy to early lactation implicate them in lactogenesis while the *in vitro* study discussed previously related the histone changes to the hormones known to be required for initiation of casein synthesis.

Histones are also known to bind some steroids. Hydrocortisone was shown to bind, *in vitro* and *in vivo*, to a histone fraction from rat liver. The greatest hydrocortisone binding was present in the arginine-rich fraction (66,67) while testosterone bound preferentially to the lysine-rich histone fraction (68). The arginine-rich histone molecule has a hydrophobic region which was suggested to have a relatively larger hydrocortisone-binding capacity than the remainder of the molecule (67) and may account for its preferential binding of hydrocortisone.

Binding Studies

Transcortin, CBG

The earliest studies of steroid binding were performed on blood. An α-globulin in blood has a high affinity for hydrocortisone and corticosterone (see 6). This α-globulin has been called corticosteroid-binding globulin (CBG) (69) or transcortin (70) and it is a 4S protein of 52000± 1500 molecular weight which binds one molecule of steroid per molecule of protein. CBG has an association constant for hydrocortisone of 3×10^{-8}M and 2×10^{-9}M at 37°C and 4°C, respectively, but it will bind progesterone more tightly than hydrocortisone at 37°C (6).

Progesterone is among those steroids highly competitive with corticosterone for human transcortin and cortisone, estradiol, and testosterone are among those steroids found to be moderately competitive (71).

Concentrations of hydrocortisone and corticosterone bound in the peripheral circulation of a number of mammalian species are given in Table 1. The cow has considerably less bound steroid than the other species.

TABLE 1.--Bound hydrocortisone and corticosterone in the peripheral circulation (modified from reference 6).

Species	Hydrocortisone		Corticosterone	
	μg/100ml	X 10^{-7}M	μg/100ml	X 10^{-7}M
Rabbit	20.0	5.5	23.0	6.6
Rat	45.0	12.4	52.0	15.0
Guinea pig	20.0	5.5	11.0	3.2
Cow	5.0	1.4	4.0	1.2

CBG binding activity and blood glucocorticoid levels have been related to pregnancy and lactation in a number of species. In the mouse, blood corticosterone levels increased during pregnancy then dropped sharply after parturition to a low at four days of lactation. CBG activity followed the same pattern but appeared to drop more sharply than total blood levels (7). In the rabbit, blood hydrocortisone and corticosterone concentrations decreased slightly between nonpregnancy and pregnancy but with the onset of lactation both increased, with hydrocortisone increasing more than corticosterone. As in the mouse, CBG activity increased during pregnancy and dropped after parturition (7). Greater decreases in CBG concentration than in total glucocorticoid concentration should yield increased free glucocorticoid. Unbound serum corticosteroid levels in rats, mice, and rabbits during pregnancy and lactation are shown in Table 2 (from Gala and Westphal, 7, 72). Each species showed a considerable increase in free steroid at the onset of lactation and further increases during lactation.

TABLE 2.--Unbound serum corticosteroid levels (μg/100ml) during pregnancy and lactation (7, 73).

Stage of pregnancy or lactation	Species and corticosteroid		
	Rat (Corticosterone)	Mouse (Corticosterone)	Rabbit (Hydrocortisone)
Nonpregnant virgin	0.05	0.39	1.14
Midpregnant	0.18(14)*	0.12(12)	2.66(21)
Late pregnant	0.26(20)	0.48(18)	0.51(27-28)
Early lactation	0.60(3)	0.74(1)	2.06(1-2)
Late lactation	1.02(12)	1.16(14)	2.59(6-12)

*Day of pregnancy or lactation.

Estrogen in Target Tissues

Much of the work concerning steroid binding in target tissues has involved the binding of estrogen. Jensen and Jacobsen (73), studying estrogen binding, have been credited (74) with obtaining the best early evidence that target tissues contain molecules which specifically interact with hormones and subsequently modify biological function. They injected estradiol into immature rats and showed the hormone to be specifically concentrated in the uterus and vagina. A number of other tissues in various species will also bind estrogen. Jensen et al. (75) cite reports of high estrogen affinity in rat, mouse, sheep, and goat uterus and vagina; human uterus and mammary tumors and rat anterior pituitary and mammary tumor. Rat liver and mammary gland also show such affinity (76).

Numerous studies have localized the binding and considered the binding kinetics (76,77). Shyamala and Gorski (74) showed that the binding of 17 β-estradiol in the rat uterus was first associated with a cytoplasmic 9S protein, of at least 100,000 MW (78). The estrogen then moves into the nucleus where it is bound to an acidic (79) 5S protein which can be extracted from the chromatin.

As the estrogen moved into the nucleus the 9S protein disappeared or lost its ability to bind estradiol. The authors suggested that the estrogen changed the conformation of the 9S cytoplasmic receptor protein enabling it to subsequently move into the nucleus (74).

Glucocorticoids

Investigations of glucocorticoid binding have been performed on a number of tissues, both *in vivo* and *in vitro*. *In vivo* binding is usually studied after corticosteroid injection of rats. Injected corticosterone is taken up by a number of rat tissues (80). The quantities of corticosterone bound in various subcellular fractions of several of those rat tissues are shown in Table 3. Liver was the only tissue to concentrate glucocorticoid above blood levels. Subcellular distribution of bound glucocorticoid was studied in liver from glucocorticoid injected normal and adrenalectomized rats. The mitochondrial fraction bound more hydrocortisone or corticosterone than the nuclear (80,81), microsomal or supernatant (87) fractions and *in vitro* much more corticosterone than hydrocortisone was bound (82). Binding

TABLE 3.--Mean subcellular distribution of the corticosterone-H^3 fraction in rat brain, thymus, heart and liver 30 minutes after an intravenous injection of 20 microcuries of corticosterone-H^3.

Cell fraction	Tissue			
	Brain 3*	Thymus 4	Heart 4	Liver 4
Nuclei	14.4**	13.9	15.2	23.8
Mitochondria	6.4	6.8	3.1	6.5
Microsomes	4.6	4.0	6.1	14.4
Supernatant	69.0	76.5	75.7	48.0

*Number of rats.
**Activity expressed as percent of total activity contained in the homogenate.

in the microsomes and mitochrondia has also been shown by others (83,84).

In order to determine whether the mitochondrial binding material was an integral part of the structure, rat liver mitochondria were sonically disrupted and centrifuged 105,000 x g. Forty-nine percent of the total mitochondrial protein was sedimented and that 49% of the protein contained 95.5 and 91.8 percent, respectively, of hydrocortisone and corticosterone bound to the mitochondria. In those mitochondria more corticosterone than hydrocortisone was bound per mg protein (81).

The binding in the 100,000 x g supernatant fraction from rat liver labeled in vivo appeared in the Sephadex G-100 and G-50 exclusion peaks (83,85,86,87,88); however, exclusion peak material prepared from rat liver would not bind hydrocortisone in vitro (86). The glucocorticoid bound to liver in vivo has been suggested to be a modified hydrocortisone (86) and two cytoplasmic proteins able to bind hydrocortisone metabolites have been isolated (89).

The number of Sephadex-separable corticoid-binding macromolecules has been studied in pigs and rats. Pigs injected with hydrocortisone-H^3 showed two labeled

cytoplasmic macromolecular peaks eluted from G-100 in liver and spleen but only one such peak in thymus (90). Rats showed the two peaks in liver and one in thymus (91). In the pigs, as in the rats, only the liver concentrated hormone above blood levels (90).

Glucocorticoid binding has also been studied in thymus cells. Thymus cells labeled with various glucocorticoids *in vitro* yielded a rapidly labeled physiologically saturable binding fraction and many of the steroids competed for binding in proportion to their glucocorticoid activity (92). At least fifty percent of the glucocorticoid bound in those cells labeled *in vitro* was in the nucleus (93,94) and was extractable with 0.6 M KCl, pH 8.0 (95). The steroid bound in rat thymus cells, in contrast to liver, was hydrocortisone and not some modified hydrocortisone molecule (92).

Some tissues which did not bind any significant amounts of glucocorticoid were rat skeletal muscle (82), heart and brain (91) and pig heart muscle (90).

Most of the tissue binding measurements have neglected mammary gland. However, in view of recent work concerning the initiation of casein synthesis in mammary explants there has been renewed interest in mammary

glucocorticoid binding. In one limited study, the amount of corticosterone bound increased in both the nuclear and extranuclear fraction with the onset of lactation in rat mammary tissue; however, very little increase was shown between pregnancy and lactation in bovine tissue (96).

Mammary glucocorticoid binding has also been studied in bovine mammary cell cultures. Mammary cells cultured with 2-16 X 10^{-12}M labeled hydrocortisone showed 34% of the incorporated label in the nuclear fraction while the 15,000 x g and 105,000 x g precipitates contained 2 and 1% of the label, respectively (97,98). The association constant for hydrocortisone was reported to be about 10^{-9} - 10^{-8}M (98). Hydrocortisone uptake and binding was interfered with by progesterone, but not diethylstilbestrol or 17 β-estradiol (97,98).

General Techniques

The researchers cited have used many ways to measure binding of steroid to particulate and macromolecular cell fractions. In vivo incorporation was measured with animals injected with radioactive steroid. Those animals were killed after a specified time and their tissues

removed, rinsed, and fractionated, usually by homogenization and differential centrifugation. The radioactivity incorporated into the various fractions was then measured. Binding in the 100,000 x g supernatant, or cytoplasmic fraction, was generally measured by molecular seive chromatography. Binding measurements in cells grown in culture were usually performed in similar manner after incubation of the cells with labeled steroid.

Incubation with steroid is frequently done after the tissues and cells are disrupted and fractionated. A homogenate can be incubated with radioactive steroid and the labeled particulate fractions then centrifugally separated or the particulate fractions can be separated and then individually incubated with steroid and measured for binding capacity.

In vitro work is frequently concerned with the physiochemical aspects of steroid binding. Effects of pH, temperature, and steroid concentration are often of concern. The most frequently used method of measuring binding under various conditions is equilibrium dialysis. Ultrafiltration can also be used because both equilibrium dialysis and ultrafiltration allow measurement to be made at equilibrium steroid concentrations.

Other methods of measuring binding after incubation with steroid are subsequent passage through a molecular seive column or precipitation with ammonium sulfate. These two methods are frequently used to separate bound from unbound steroid and hence allow measurement of bound steroid. Both methods give more equivocal results than equilibrium dialysis or ultrafiltration. The results are more equivocal because elution through a molecular seive exposes the steroid-binding molecule to a zero steroid concentration which encourages dissociation of the steroid and ammonium sulfate precipitation aggregates and denatures protein which could affect binding capacity and tenacity even though the denaturation may be reversible.

Kinetic studies of binding dynamics relate degree of binding to amount of steroid present. Data collected in such studies can be plotted in a manner similar to enzyme data. Lineweaver-Burk-type graphical plots (see 99) of the reciprocal of bound steroid vs the reciprocal of steroid concentration will yield binding constants and number of binding sites, respectively equivalent to K_M and V_{max} in enzyme studies. The y intercept is the reciprocal of n, the number of binding sites, the x intercept is the negative reciprocal of k, the binding

constant, and the slope is -1/kn. Scatchard, in justifying a "better" method to plot binding data states that the double reciprocal plot has the disadvantage of concealing deviations from ideal laws and tempting straight lines where there should be curvature (100).

Scatchard's method is the preferred method for plotting binding data. His plot of bound: free vs bound ligand gives a straight line if k is constant. The y (bound:free) intercept is -kn and the x intercept is n; therefore, the slope equals -k.

Often there are two or more binding sites, each with different affinities for steroid, within a mixture or molecule. To remove the influence of one binding site on another further treatment of the Scatchard plot can be performed (71). The portion of the curve due to low affinity binding is geometrically subtracted from the high affinity binding curve supposedly leaving a pure high affinity curve. However, as the high affinity portion of the binding curve merges with the low affinity portion, subtraction of points in the area of the merging curves will cause the derived line to bend back to the origin. Such geometrical subtraction has recently been used in a study of corticosterone binding in rat liver (84) but

most data in the literature is usually derived solely from a Scatchard plot.

Time and Temperature Effects

Most of the studies dealing with the time course of glucocorticoid uptake have used rat liver and thymus. In rat liver slices, uptake of hydrocortisone followed Michaelis-Menten kinetics with a V_{max}, at 4°C, of 9×10^{-11} moles/min/g wet tissue and a K_m of 2.7×10^{-7}M (101). Diffusion was thought to control the rate of uptake in those rat liver slices since equilibrium was reached in 6 hours with both 10^{-9}M and 10^{-6}M corticosterone at 0°C (84).

The time required to achieve equilibrium has been related to the steroid concentration by incubation of hepatoma cells with two steroid concentrations. The hepatoma cells incubated with 10^{-6}M glucocorticoid showed maximum labeling of binding sites in both nuclei and supernatant in less than 5 min while at a lower concentration, 5×10^{-9}M steroid, the supernatant binding sites required 5 min and the nuclei 30 min to become saturated (57).

The time course of hydrocortisone transfer from cytoplasm to isolated nuclei has been studied in rat liver. Cytosol was isolated and labeled with radioactive hydrocortisone. Rat liver nuclei were incubated with the labeled cytoplasmic binder and within 10 min were maximally labeled. The nuclei incubated with the labeled cytosol fraction bound 1.2 times more label than nuclei incubated with either free hydrocortisone or hydrocortisone plus albumin (88).

The effect of temperature on binding kinetics has also been examined. At 37°C rat liver cytosol within 6 min bound 16% of available hydrocortisone but after 10 min there was a rapid decrease in amount of steroid bound. At 17°C and 4°C more of the available steroid, 21 and 26%, respectively, was bound but the incorporation was much slower; 95 min were required to reach maximum binding at 17°C and 200 min at 4°C (88).

Dissociation has also been examined in thymus cells diluted 50-fold after incubation with 6×10^{-9} M hydrocortisone. Those cells showed a rapid, marked dissociation of hydrocortisone. The cells lost two-thirds of the bound steroid in about 20 min after dilution at 3°C. After the rapid loss of steroid, the rate of

dissociation decreased but rapid dissociation occurred again after warming the suspension from 3°C to 37°C (94). The dissociative properties of hydrocortisone in the thymus cell have been used to distinguish high and low affinity binding in those cells. From the high affinity sites hydrocortisone dissociated slowly, as a first order process, with a half time of dissociation of less than 3 min at 37°C while the low affinity sites had a dissociation half time of less than 15 sec (92,94).

MATERIAL AND METHODS

Tissues

Most of the work done in this study was performed on lactating bovine mammary tissue; however, some experiments were performed with lactating rat mammary tissue. All of the bovine tissue was obtained from an abattoir but was subjected to different treatment depending on whether it was to be stored frozen or fractionated before storage. Bovine tissue was always removed from the udder within 20 min of death. Rat tissue was removed immediately following decapitation. If the tissue was to be frozen whole, it was rinsed in 0.15 M KCl, placed in a polyethylene bag and kept on ice until it was frozen at -55°C. In one case, mentioned in the Results and Discussion section, the bovine tissue was rinsed with KCl containing penicillin and oxytocin which helped remove residual milk from the tissue. Tissue to be fractionated before storage was removed and washed with an ice-cold buffered salt solution (TMK) containing tris (2-amino-2-(hydroxymethyl)-1,3-propanediol) (0.01 M, pH 7.2),

$MgCl_2$ (0.0015 M) and KCl (0.01 M), to remove as much milk as possible.

Homogenization

Tissues were minced, diluted with three or four volumes of cold TMK and homogenized either with a glass tube-teflon pestle homogenizer driven by an electric drill, or with a Willems Polytron Model PT10 (Brinkmann Instruments Inc., Westbury, New York) run at medium speed until no pieces of tissue were visible. The homogenate was filtered through four thicknesses of cheesecloth to remove connective tissue if the 750 x g precipitate was to be used for experimental purposes. If the 750 x g precipitate was to be discarded, the homogenate was not cheesecloth filtered but the connective tissue was sedimented and discarded with the 750 x g fraction.

Tissue Fractions

The initial fractions studied were obtained by differential centrifugation. The low-speed fractions were obtained at either 600 or 750 x g. The 27,000 or 30,000 x g fractions were the precipitates resulting from 27,000

or 30,000 x g centrifugation of the 600 or 750 x g supernatant. Thirty-thousand x g precipitates referred to in this dissertation were produced by 30,000 x g centrifugations of whole homogenate. The centrifugations at 30,000 x g or less were performed in a Sorvall RC2B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). The 100,000 x g precipitate of the 27,000 or 30,000 x g supernatant, termed the 100,000 x g fraction, was produced in a Spinco Model L centrifuge (Beckman, Palo Alto, California) by a 90 min centrifugation in a number 50 rotor.

Any of the supernatants used for experimental purposes are referred to in the Results and Discussion section as supernatants of the given centrifugation in order to avoid confusion with the particulate fraction produced by that centrifugation.

Supernatants of the 750 and 30,000 x g centrifugations were usually passed through a glass wool plug in a glass funnel in order to remove as much lipid as possible before the next centrifugation or any other fractionation. Particulate fractions produced by the centrifugations were washed by addition of cold TMK, disruption of the pellet and recentrifugation.

The above pellet fractions were further fractionated by extraction with 0.1 N NaOH. The final standard procedure for NaOH extraction was the addition of 0.5 ml of 0.1 N NaOH per 10 ml of original 750 x g supernatant. The pellet was disrupted, extracted for 30 min and then recentrifuged after dilution with an equal volume of cold distilled water and used fresh or stored frozen at -10°C.

The above supernatants were further fractionated by ammonium sulfate precipitation. The ammonium sulfate precipitations were made on a volume to volume basis using cold saturated ammonium sulfate adjusted to approximately pH 7.2 [measured with Accutint (Anachemia Chemicals, Ltd., Champlain, New York) or pHydrion (Micro Essential Laboratory, Brooklyn, New York) pH paper] with ammonium hydroxide. The precipitates were allowed to form for 20 min while stirring in the cold. The suspension was then centrifuged 5 min at 30,000 x g and the pellet produced was redissolved in a volume of 0.1 N NaOH corresponding to 0.01 the volume of the starting supernatant (750 x g or 30,000 x g supernatant of NaOH extract). An equal volume of TMK was then added and the solution dialyzed against TMK to remove residual ammonium sulfate. The ammonium sulfate fractions are named by the

percentages of saturated ammonium sulfate which produced them. They are the precipitates, or resuspended precipitates, obtained by making a solution to the given percentage with saturated ammonium sulfate. Unless specified otherwise, all the ammonium sulfate fractions were produced from the 30,000 x g supernatant of the mammary tissue homogenates.

Molecular seive chromatography [Sephadex G-100, 40-120 mesh (Pharmacia Fine Chemicals, Piscataway, New Jersey)] was used to separate bound and unbound steroid and for further fractionation of ammonium sulfate fractions. Glass columns used varied in size from modified Pasteur pipettes to a Pharmacia 1.5 x 30 cm column. The columns were eluted with distilled water at room temperature and timed fractions were collected on a fraction collector.

Calcium phosphate gel fractionations were performed with 94-96% moisture gels prepared by published procedures (102). The gel was stored and used in the cold. Fractionations by calcium phosphate were performed in two different ways. One way was addition of a given quantity of gel to the material being fractionated. After incubation, the gel was centrifugally removed from solution and

a larger quantity of fresh gel added to the solution. After each addition of gel the mixture was stirred frequently for 20 min in a 15 ml conical glass centrifuge tube. This procedure was repeated with increasing quantities of gel after which the gels were each extracted with a K_2HPO_4 solution of given molarity and pH.

Calcium phosphate gel fractionation of the ammonium sulfate fractions was performed differently. Gel was added at 1 g (wet weight) gel per 1-3 ml dialyzed ammonium sulfate fraction and incubated 20 min in the cold. It was then sedimented at 2,000 RPM in an International Model V centrifuge (International Equipment Co., Boston, Mass.) and washed 1-3 times with 1 ml 1 mM K_2HPO_4, pH 7.4, per g gel added. After washing, the gel was extracted with similar volumes of K_2HPO_4 of higher concentration and pH as given with the results.

Cellex-D (Bio-Rad Laboratories, Richmond, California) was used for DEAE-cellulose fractionations. It was equilibrated with cold distilled water and packed in 0.8 X 30 cm glass columns to about 10 ml volume. Columns were prepared and eluted at 4°C. Samples to be fractionated were placed on the column and the column then rinsed with 1 mM K_2HPO_4, pH 7.4. The fractions were eluted from

the column by increasing concentration and pH K_2HPO_4 as given in the Results and Discussion section.

Steroid Binding

Steroid binding experiments to measure distribution of binding among cellular fractions were usually performed with the tissue homogenate diluted 1 g to 4 ml of TMK, except for two experiments when the homogenate was diluted 1 g to 9 ml of TMK. All incubations were performed in either plastic or Corex (Corning Glass Works, Corning, New York) centrifuge tubes and labeled steroids were dissolved in ethanol and added to the mixture with a 10 or 50 µl syringe. Ethanol concentrations in the incubation mixtures were normally less than 1% with a maximum of 2% and agitation during addition of the steroid in ethanol ensured complete mixing. When the incubation mixtures were used to measure incorporation of steroid into particulate fractions the incubations were of 15-60 min duration at 0°C, 4°C or room temperature.

Radioactively labeled steroid hormones (New England Nuclear, Boston, Mass.) used in the various incubations included corticosterone-1,2-H^3 (30 Ci/mM),

corticosterone-4-C^{14} (55 mCi/mM), hydrocortisone-4-C^{14} (51.8 mCi/mM), progesterone-4-C^{14} (52.8 mCi/mM) and 17 β-estradiol-C^{14} (47.6 mCi/mM).

After incubation with any of the labeled steroids the incubation mixture, or portions of it, were subjected to washing with buffer or Sephadex, charcoal or dialysis treatments. These treatments were attempts to remove unbound steroid from the incubation mixture. The buffer washes and Sephadex treatments are discussed in the Results and Discussion section. One of the other methods, charcoal treatment, was performed by layering either Darco (Atlas Chemical Industries, Wilmington, Delaware) or Norit 1 (Sigma Chemical Co., St. Louis, Missouri) charcoals on 25 mm diameter filters (Millipore Filter Corp., Watertown, Mass. Type SC 84). The charcoal was suspended by rapid stirring and an aliquot of the suspension delivered onto the filter. The filters were held in a Millipore filter apparatus on a vacuum flask. Incubation mixture could then be drawn through the charcoal layer by vacuum and delivered into a small test tube inside the vacuum flask. The charcoal used on the filters was in some cases coated to reduce adsorption of protein from the incubation mixture. Coatings used were dextran

(60,000-100,000 MW) and polyvinylpyrrolidone (PVP). Amounts of these two materials used for coating the charcoal are given with the results and discussion of the experiments in which they were used.

Charcoal was also combined with dialysis to separate unbound from bound steroid. The incubation mixture (5 to 8 ml) was dialyzed against 300-600 ml TMK containing 1-2 g activated charcoal kept in suspension by magnetic stirring. Either 1 or 2.5 cm flat width Visking tubing was used for the dialyses which were done at 4°C.

Equilibrium dialysis was also used for binding studies. The first equilibrium dialysis experiments were performed with 1 cm Visking dialysis sacs suspended in 500 or 600 ml beakers containing TMK and the steroids being tested. Later equilibrium dialysis experiments, used to determine binding competition and constants, used 1 cm sacs containing 2 ml ammonium sulfate fraction. Those sacs were suspended in TMK to a total volume of 100 ml in a 110 ml test tube. Three to four tubes, each with a different steroid concentration, were used to study degree of binding with varied steroid concentration. Attainment of equilibrium in these experiments was monitored by suspending sacs containing only 2 ml of TMK in

the low and high steroid concentration tubes. All of these equilibrium dialysis experiments proceeded about 12 hours with constant magnetic stirring.

The final series of binding constant determinations also used equilibrium dialysis and the 60-80% saturated ammonium sulfate fraction, however, twelve, rather than three or four, steroid concentrations were used and the dialysis time was 24 hours rather than 12.

Other

Measurements of radioactivity were performed by the liquid scintillation method using a Nuclear-Chicago Model 720 liquid scintillation spectrometer (Nuclear-Chicago, Des Plaines, Illinois). The liquid scintillation fluid contained dioxane, xylene, ethanol, naphthalene, 2,5-diphenyloxazole and 1,4-bis-(2-(4,methyl-5-phenoxazolyl))-benzene (Appendix 1). Vials used to contain the scintillation mixture were either polyethylene or glass and 10 ml of the scintillation fluid was added to one half ml of aqueous sample to be counted.

Electrophoresis was performed on 7% polyacrylamide gels made according to a Canalco formulation (Appendix 2).

The stacking gel was pH 8.9 and the running gel pH 9.5. The gels were electrophoresed in a Buchler apparatus (Buchler Instruments, Inc., Fort Lee, New Jersey). Samples placed on the gels had sucrose added to increase their density and were delivered to the top of the gel with a microsyringe (Hamilton Co., Whittier, California). Five to 80 μl samples were applied to the gels. In order to visibly observe the electrophoresis in the gels bromphenol blue solution (1 ml of 0.005% solution) was added to the electrophoresis buffer as a tracking dye. The electrophoresis started at 1.25 milliamps (mA) per tube for 20-30 min until the sample entered the running gel and then the amperage was increased to 2.5 mA per tube. The electric current was stopped when the tracking dye neared the end of the gel after which the gels were removed from the tubes and stained with Buffalo (amido) black in 7.5% acetic acid. After 8-12 hours in the stain they were destained by reverse electrophoresis in 7.5% acetic acid. After destaining the distances traveled by the bands of protein were measured relative to the distance traveled by the tracking dye. The relative distance moved was called the R_{dye} (R_d) value.

The one immunodiffusion study was done using Ouchterlony agar diffusion plates (103) made with 0.85% agar in 0.85% saline buffered with 0.005 M phosphate, pH 7.4. Antibodies used on the plates were produced by two rabbits injected subcutaneously twice, 11 days apart, with 50-80% saturated ammonium sulfate fraction and again 31 days later with 60-80% saturated ammonium sulfate fraction mixed 1:1 with Freund's adjuvant. The rabbits were bled from the ear two months after the first injection; 13 days after the third injection. Serum was prepared from the blood and the serum was stored cold with 0.0001% merthiolate added.

Determination of protein concentration were performed by 260/280 absorbance ratios and the method of Lowry et al. (104). The absorbancies for the 260/280 ratios were read in a Gilford spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) and the protein concentrations determined from those ratios by reference to a nomograph prepared by the California Corporation for Biochemical Research.

RESULTS AND DISCUSSION

Measurement of Binding

The quantity of steroid which will bind to some material is usually determined by binding a radioactively labeled steroid to that material and subsequently measuring the amount of label bound. The quantity of steroid bound can be found by difference if bound plus unbound label are measured and if unbound, or free, label can be determined in some manner. Such is the case in equilibrium dialysis where bound plus free label is measured inside a dialysis sac and free label is measured in the medium surrounding sac. More frequently, however, bound label is determined by procedures which separate bound from free steroid.

Dilution Methods

One method for separation of bound and free steroid is extensive washing which will dilute the free steroid away from the bound steroid. This method is

particularly suited for use on particulate fractions. Another method often used is molecular seive chromatography, or gel filtration. Generally, Sephadex G-100 or G-50, or their equivalent are used to allow exclusion of the macromolecular binding protein and retarded passage of the free steroid. The method is well suited for use with soluble binding proteins. However, both extensive washing and gel filtration have a distinct disadvantage to their use for measuring bound steroid. In both cases the wash or elution media have no steroid in them, i.e. steroid concentration is zero and dissociation occurs.

One of the earliest experiments in this study indicated dissociation during pellet washing and gel filtration. Although the experiment was not designed to demonstrate dissociation during washing and gel filtration, calculations of the amount of label added to the incubation mixture versus that recovered as bound and free steroid did indicate the possibility that such dissociation was occurring. Those calculations showed that the increased unbound steroid measured after washing and gel filtration would probably not be attributable to contaminating supernatant from the original incubation mixture.

The experiment from which the data was calculated was performed by adding corticosterone-H^3 (2.2×10^6 dpm) to 2 ml of a resuspended 27,000 x g precipitate from bovine mammary gland. After that incubation with labeled steroid, the suspension was recentrifuged. The pellet was carefully separated from the supernatant and suspended in 1 ml of phosphate buffer containing 0.05 N NaOH. An aliquot of that suspension was passed through Sephadex G-100 and the Sephadex fractions counted. Counting efficiency, normally about 10%, had to be assumed so in order to obtain a minimum contamination value, a maximum counting efficiency (20%) was assumed. Using that 20% counting efficiency there were 1.6×10^5 dpm from unbound corticosterone after passage through G-100. For that many dpm to be unbound, 7.5%, or 0.15 ml, of the original 2 ml of incubation mixture would have to have been contaminating the pellet after the post-incubation centrifugation. That much contamination could not have been present and the data were considered to represent dissociation occurring during resuspension and elution through Sephadex.

Dissociation occurring during gel filtration has been observed in binding studies reported in the literature. Fiala and Litwack (86) considered the extent of

dissociation which will occur on Bio-Gel P-100, which is similar to Sephadex G-100. After passage through P-100, 48% of the total hydrocortisone added to serum in vitro remained bound compared to 80% during ultrafiltration, in which the free steroid concentration remains unchanged. Similarly, ultrafiltration showed that particle-free supernatant from livers of hydrocortisone-injected rats bound 50% of the steroid but gel filtration showed only 13% bound (86).

Dissociation might also occur during washes of particulate fractions. This was indicated in an experiment using homogenates incubated with four levels of corticosterone. Four successive washes of the 750 plus 27,000 x g fractions obtained from those homogenates gave the data shown in Table 4. The data, expressed as percent of total recovered radioactivity, show that most of the label which appeared in Wash 1 was probably contaminating supernatant. In Wash 2 some of the label could also be contaminating supernatant, but the label in Washes 3 and 4 probably represent dissociation of bound label from the pellet being washed. Dilution should reduce the label in Wash 4 drastically but although the label in Wash 4 fell below that of Washes 2 and 3, it does not reach the low

TABLE 4.--Percentage of total recovered label found in each of four washes of 27,000 x g precipitates from homogenate incubated with four levels of corticosterone.

Wash	Initial steroid concentration X 10^{-9} M			
	0.83	1.66	3.32	4.98
1	4.49	4.78	5.36	5.53
2	0.71	0.85	0.89	0.85
3	0.76	1.26	1.01	0.99
4	0.61	0.60	0.44	0.61

level which would be expected if there was no dissociation occurring.

Adsorption Methods

Another method for separation of bound and free steroid is the use of some adsorbant with a strong affinity for steroid. The adsorbant is usually added to a mixture of bound and free steroid, the free steroid adsorbed and then the label not adsorbed, representing bound steroid, is measured. Two adsorbants commonly used in steroid-binding studies determinations are Florisil, an activated magnesium silicate, and charcoal. Both materials have been coated with dextran (105,106,107), to reduce protein adsorption. A number of determinations during this project showed the adsorption methods to be of little value to this study.

Florisil was first tested with a NaOH extract of the 27,000 x g fraction. The NaOH extract was incubated with corticosterone-H^3 and then passed through G-100. The radioactivity recovered from the G-100 showed a total corticosterone concentration of about 8.3×10^{-13} moles in 0.2 ml with 37% of that bound to a macromolecule. To

0.2 ml of the same labeled NaOH extract 24 mg of Florisil was added and the solution passed through G-100. The amount of steroid bound was unaffected while the free steroid was reduced to only 71% of its pretreatment level. A 29% reduction in free steroid did not justify the routine use of Florisil to remove free corticosterone-H^3 from solution.

Next, the removal of steroid from buffer was studied to eliminate any effect of the NaOH (high pH) used in the first experiment. Regular and heat-activated Florisil were added to TMK containing corticosterone-H^3. The Florisil was mixed with the solution and allowed to sit for one hour at room temperature. Aliquots of that solution were counted and the data obtained is shown in Table 5. The data of Trapp and West (106), included in the table for comparative purposes, was obtained by mixing hydrocortisone in a phosphate buffer with dextran-coated Florisil and shaking one hour at 4°C. Under these conditions, 80 and 90% of the steroid in a given volume was adsorbed by 40 and 80 mg Florisil, respectively (106). In contrast, the plain Florisil used in this study, which presumably should bind more steroid than dextran-coated Florisil, bound only 23 and 62% of the available steroid

TABLE 5.--Amounts of glucocorticoid adsorbed by Florisil and comparison with Trapp and West (106) data.

Steroid	Florisil		Volume*	Steroid concentration	Steroid adsorbed
	Type	Quantity			
		(mg)	(ml)	(X 10^{-10} M)	(X 10^{-13} moles)
Corticosterone	Regular	15.6	1.5	2.7	0.93
		300	1.5	2.7	2.55
	Heated	40	4.0	2.7	0.96
Hydrocortisone (Trapp and West)	Dextran-coated	40	0.5	4000	1600
		80	0.5	4000	1800

*Volume of reaction mixture.

on 15.6 and 300 mg, respectively. The dextran-Florisil, used by Trapp and West (106), although it was removing such a high proportion of steroid, was leaving a higher concentration, 400 X 10^{-10} M, of unbound steroid than that used in this study as the initial concentration. Therefore, Florisil may not be able to adsorb much steroid from the solutions of very low steroid concentration used in this study. Because of that lack of high affinity binding in Florisil higher proportions of unbound steroid are left in solution and the method becomes unsuitable for use in this study of high affinity binding from dilute steroid solutions.

The efficiency of adsorption methods and the desire to utilize them for faster analyses led to experiments with other adsorbants. In one experiment, 30 mg granular activated charcoal added to 1 ml TMK containing 1.66 X 10^{-10} M corticosterone removed 97% of the free steroid within five minutes. In another experiment dextran-coated (0.0125 g activated charcoal, 0.0015 g dextran) charcoal also showed rapid removal of steroid from solution. The coated charcoal was centrifuged out of suspension but usually contaminated the end of the

pipette used to withdraw a sample. This contamination led to highly erratic data.

A relatively large amount of steroid in the 50-80% saturated ammonium sulfate fraction went through the charcoal filter on first passage and should represent the large amount of steroid-binding protein present in that fraction. That observation stimulated further interest in the highly (over 50%) saturated ammonium sulfate fraction and further study of that fraction will be reported in other sections.

Coating of charcoal is a means of minimizing protein adsorption to the charcoal. Three different coated charcoal preparations were tested in this study (Table 7). One ml of each coated-charcoal suspension was placed on a Millipore filter. Test solutions were the NaOH extracts of 750 x g and 30,000 x g fractions and resuspended 0-50 and 50-80% saturated ammonium sulfact fractions (from 30,000 x g supernatant). These were incubated with corticosterone and passed through the filters. In Table 7 both protein and label recovery after passage through dextran-coated charcoal are expressed as percent of the original amount.

TABLE 6.--Adsorption of corticosterone passed through 0.1 g activated charcoal on a 8 μ Millipore filter.

Carrier	Volume (ml)	Steroid concentration (X 10^{-10} M)	Steroid removed (% of initial)
TMK	3	3.3	95.5
266 μg BSA in TMK	4.5	1.1	99.7
750 x g NaOH extract			
1st passage	6	2.7	98.2
2nd passage	6	2.7	99.1
30,000 x g NaOH extract	6	2.7	98.2
30,000 x g supernatant			
0-50% ammonium sulfate fraction	6	2.7	97.4
50-80% ammonium sulfate fraction			
1st passage	6	2.7	91.5
2nd passage	6	2.7	97.4

TABLE 7.--Loss of corticosterone and protein by passage through three coated-charcoal preparations on a 8 μ Millipore filter.

Fraction*		Initial concentration	Dextran-Darco** filtrate	PVP-Norit 1*** filtrate	Dextran-Norit 1**** filtrate
			% of original		
750 x g NaOH extract	steroid	2×10^{-10} M	5.0	0.8	3.4
	protein	.995 mg/ml	6.2	22.4	9.5
30,000 x g NaOH extract	steroid	2×10^{-10} M	1.2	0	3.7
	protein	1.143 mg/ml	2.5	21.1	13.8
30,000 x g supernatant 0-50% ammonium sulfate fraction	steroid	2×10^{-10} M	0	0	2.7
	protein	2.929 mg/ml	12.9	2.2	---
50-80% ammonium sulfate fraction	steroid	2×10^{-10} M	1.4	0.6	15.7
	protein	1.336 mg/ml	15.7	45.8	44.0

*Two ml of each fraction passed through charcoal layered on filter.

**Three-tenths g Darco S51 charcoal, 0.31 g dextran, 10 ml TMK; 1 ml on filter.

***Five g Norit 1 charcoal, 1% PVP, 20 ml TMK; 1 ml on filter.

****Twenty-five hundredths g Norit 1 charcoal, 0.03 g dextran, 10 ml TMK; 1 ml on filter.

Some steroid did get through the charcoal and probably represents bound corticoid but a high percentage of protein was lost from every test solution. The very low levels of steroid recovered in the filtrates are best explained, as in the prior experiment, by loss due to protein adsorption to the charcoal.

Less protein and more steroid was adsorbed by the PVP-coated Norit than the dextran-coated Norit in all cases but all three of the coated charcoals adsorbed too much protein to be of value in routinely separating free from bound steroid. A relatively large amount of label from the 50-80% saturated ammonium sulfate fraction passed through the Norit 1-dextran. This was encouraging but the protein adsorbed to the charcoal may have contained more steroid-binding protein than was passed through.

Charcoal could not be directly used to separate free and bound steroid; however, charcoal's high affinity for steroid combined with dialysis could offer a method for good separation. The problem with using dialysis for the separation of bound vs unbound steroid is that the time required is so long that dissociation occurs and a true measurement of bound steroid is not obtained. The charcoal should adsorb any steroid dialyzing out of the

sac and would not be in contact with the protein and the dialyzing medium would remain essentially zero steroid concentration, hence, speeding dialysis. The ability of charcoal to aid in dialysis was assessed by measuring the removal of corticosterone from a TMK solution. Another control could have been a sac with corticosterone-TMK dialyzed against TMK without charcoal. However, this would only show an approach to equilibrium and would not be very meaningful since the objective was to remove all unbound steroid from the sac.

A 2.5 cm flat width Visking sac containing corticosterone-H^3 and TMK was dialyzed against TMK and suspended charcoal. The percentages of the original steroid remaining inside the dialysis sac at various sampling times are shown in Figure 1. Included in Figure 1, along with the corticosterone-TMK data, is a 750 x g supernatant fraction dialyzed under similar conditions.

Figure 1 shows the fast removal followed by a steady though slow removal of steroid after 8 hours dialysis. The slow phase was noticeable in both the buffer and 750 x g supernatant sacs. There is an obvious break in the curve at 8-9 hours and the steroid remaining

Fig. 1.--Loss of corticosterone-H^3 from solutions dialyzed against charcoal in suspension. Ten ml TMK or 750 x g supernatant containing 8.3×10^{-10}M corticosterone-H^3 dialyzed against 300 ml TMK containing 2 g charcoal in suspension.

o—o TMK

•—• 750 x g supernatant

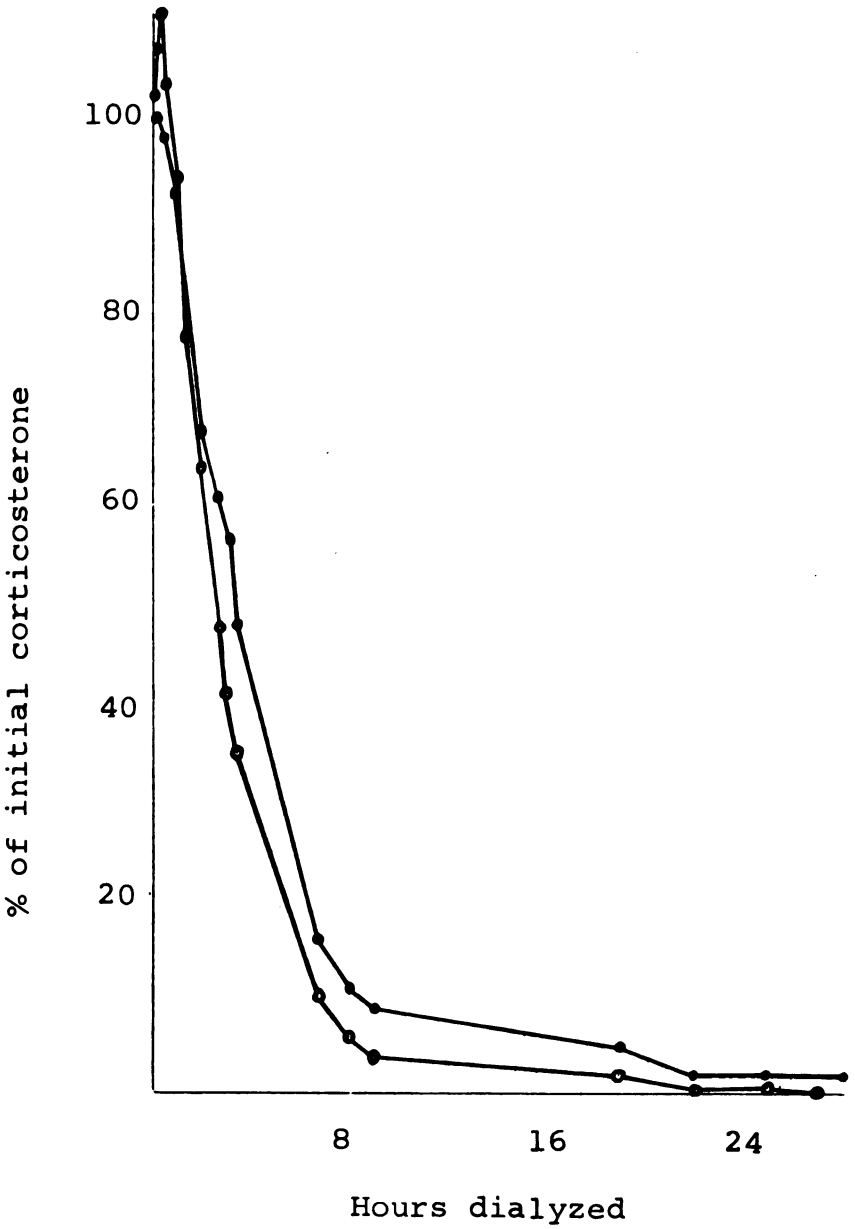
100
80
60
40
20
% of initial corticosterone
8
16
24
Hours dialyzed

Figure 1

should represent bound steroid. Eight to twelve hours of dialysis was therefore used for binding data obtained by dialysis against charcoal suspension. Two NaOH extracts and two ammonium sulfate fractions were incubated with 2.1×10^{-10} M corticosterone and then dialyzed against TMK containing activated charcoal. Samples were taken at 0, 0.5, 1.0, 7.0, and 9.0 hours and the results are shown in Table 8. After 9 hours dialysis the steroid in the fractions was assumed to be predominantly in the bound form. The 9 hour dialyzed fractions were then passed through charcoal layered on a filter to further study removal of bound steroid from solution.

Both techniques suggested binding in the 50-80% saturated ammonium sulfate fraction. Removal of label from the dialysis sac containing the 50-80% saturated ammonium sulfate fraction was slower than in any of the other fractions. When the material dialyzed 9 hours was passed through either 0.02 g Darco S51 charcoal and 0.02 g dextran or 0.02 g Norit 1 charcoal and 0.02 g dextran on a Millipore filter, all the label remaining in the dialyzed solution was removed except in the 50-80% saturated ammonium sulfate fraction. In that fraction 1.4 and 1.6% of the prefiltration label passed through the Norit- and

TABLE 8.--Removal of corticosterone by dialysis against

TABLE 8.--Removal of corticosterone by dialysis against charcoal suspension.

Fraction	Hours dialyzed				
	0	0.5	1.0	7.0	9.0
	% of original				
750 x g NaOH extract	100	96.9	76.1	10.4	4.4
30,000 x g NaOH extract	100	84.4	67.9	10.1	5.0
30,000 x g supernatant					
0-50% ammonium sulfate fraction	100	87.2	73.0	10.6	4.9
50-80% ammonium sulfate fraction	100	90.3	78.1	19.6	11.8

Darco-coated filters, respectively. If there was any binding in the other three fractions the protein was all adsorbed by the coated charcoal but the 50-80% saturated ammonium sulfate fraction either had enough binding protein so that some got through or its binding protein was less well adsorbed.

Equilibrium Dialysis

One of the most sure methods for obtaining valid measurements of extent of binding is equilibrium dialysis, although the method is somewhat time-consuming. The binding material to be tested is placed in a dialysis sac in a solution containing steroid. When equilibrium becomes established between free steroid outside the sac and free steroid inside the sac, the steroid concentration outside can be subtracted from the total concentration inside to determine the concentration of bound steroid. Dissociation need not be considered and the extent of binding at a given steroid concentration is known.

To test equilibrium dialysis 5 ml samples were dialyzed against 500 ml TMK containing 1.66×10^{-11} M corticosterone. Aliquots were withdrawn between one and

twenty hours, counted, and the data plotted in Figure 2. The data show apparent sampling error as evidenced by the variation in the buffer curve. The 0-50% saturated ammonium sulfate fraction curve showed little binding but the 30,000 x g NaOH extract showed more extensive binding with approximately the same shape as the 0-50% saturated ammonium sulfate curve. Since the curves showed only slight increases between 8.5 and 20 hours, 12 hours was chosen as the standard time for cessation of dialysis.

The 50-80% ammonium sulfate fraction curve showed a relatively large increase between 8.5 and 20 hours. The increased time required for saturation of that fraction could be a function of protein concentration in that a high concentration of binding protein in the sac would take longer to saturate, due to dialysis being rate-limiting, than a lower concentration. More dilute solutions should allow for equilibration within 12 hours.

Fig. 2.--Rates of corticosterone uptake by three cell fractions during equilibrium dialysis. Six ml of each sample with all samples dialyzed together against 500 ml TMK containing 1.66×10^{-11}M corticosterone-H^3. One half ml samples withdrawn after 1.0, 1.5, 3.5, 8.5, and 20 hours dialysis.

TMK buffer

0-50% saturated ammonium sulfate fraction

50-80% saturated ammonium sulfate fraction

30,000 x g NaOH extract

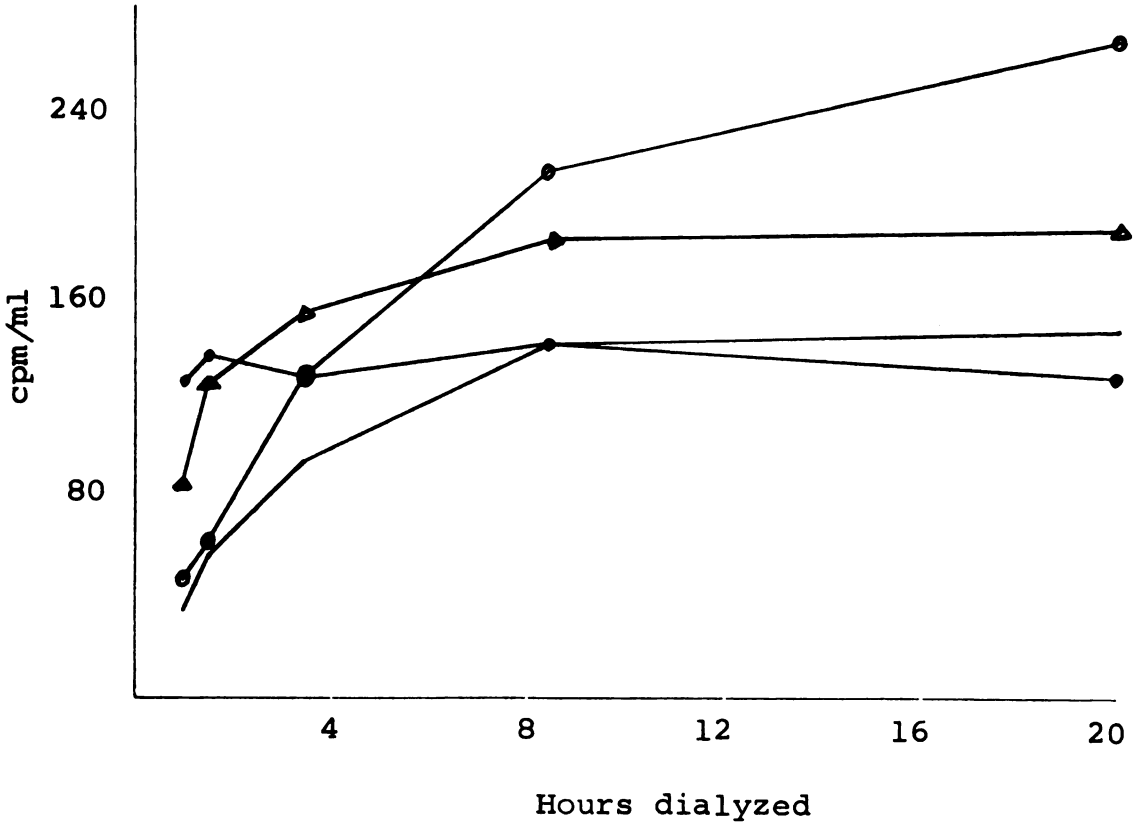

Figure 2

Isolation and Purification of Steroid-Binding Proteins

Stability

The influence of a reducing agent, mercaptoethanol, on stability of binding was determined in one experiment. Homogenates with and without 5×10^{-6} M mercaptoethanol were incubated at room temperature for 60 min in the presence of 8×10^{-10} M corticosterone-H^3. Two fractions, the 750 x g and 30,000 x g particulate fractions, were assayed for incorporated label. Duplicate control and treatment determinations gave the data shown in Table 9 as percent of total recovered label found in each fraction. The mercaptoethanol-treated material had 18 and 24% more label than the control for the 750 x g and 30,000 x g fractions, respectively. However, when the data was adjusted for total protein content of the homogenate from which the fractions came, the treated material showed 2% fewer cpm per mg protein per cpm recovered. It was concluded that mercaptoethanol did not appreciably affect binding in either the 750 x g or 30,000 x g fractions and the binding material was apparently stable without an added reducing agent.

TABLE 9.--Effect of mercaptoethanol on binding of corticosterone-H^3 to particulate subcellular fractions.

Fraction	Control	Mercaptoethanol
750 x g	1.70*	2.00
30,000 x g	1.85	2.30
CPM/mg protein CPM recovered	4.9×10^{-4}	4.8×10^{-4}

*Particulate binding expressed as percent of total recovered label.

To determine stability of the binding material during freezing and thawing, a 2 ml aliquot of resuspended 30,000 x g fraction from lactating rat mammary was incubated with 1.7 X 10^{-8} M corticosterone-H^3. After recentrifugation and extraction of the 30,000 x g sediment with 1 ml 0.05 N NaOH the extract was frozen for nine days. Some of the same extract was frozen and thawed twice and both were passed through Sephadex G-100. Thirty-seven percent of the label in the NaOH extract was bound, i.e., appeared in the G-100 exclusion peak, before freezing the extract. Frozen storage for 9 days left 33.4% of the steroid bound and the twice frozen and thawed material showed 34% bound, hence, freezing had little effect on stability of binding in the NaOH extract. Stability to freezing and thawing was also checked in the ammonium sulfate fractions prepared from the 30,000 x g supernatant. The ability of those fractions to bind corticosterone, determined by equilibrium dialysis, was not diminished by months of frozen storage or repeated freezing and thawing. Apparently the binding material was very stable during storage at -10°C.

Extraction from Particulate Fractions

Release of the binding material from particulate fractions was attempted by NaOH extraction. A 27,000 x g fraction representing 2.5 g bovine mammary tissue was "extracted" first with 2 ml 0.1 M phosphate buffer (pH 7.4), then with 2 ml of 1:1 phosphate buffer: 0.1 N NaOH. One half ml of each extract was incubated with 1.7×10^{-11} moles of corticosterone-H^3 and then passed through G-100. The macromolecular and free steroid fractions from the G-100 were counted to determine the percentage of label in the macromolecular peak, i.e., bound. Only 1.7% of the total recovered label was bound in the phosphate buffer "extract" while 7.7% of the label in the NaOH extract was bound. The four and one half times more binding seen in the NaOH extract was assumed to represent much better extraction of binding material by NaOH than pH 7.4 phosphate buffer.

Distribution of Binding Among Cell Fractions

Corticosterone binding was studied in a number of cellular fractions. Those studied were the 750 x g

pellet containing mostly nuclei, the subsequent 30,000 x g pellet containing mitochondria and microsomes, the subsequent 100,000 x g pellet, containing microsomes, and the 100,000 x g supernatant. In many cases the 100,000 x g centrifugation was neglected and the 30,000 x g supernatant used instead of the 100,000 x g supernatant.

The distribution of binding activity in particulate fractions was studied in a homogenate prepared from frozen lactating-bovine mammary gland. The homogenate was prepared in 0.1 M Tris (pH 7.5) buffer and incubated with approximately 2.5 X 10^{-8} M corticosterone. After incubation, the 600 x g, 30,000 x g, and 100,000 x g particulate fractions were centrifugally produced, washed once, and measured for radioactivity. The data are shown in Table 10 and are expressed as both the percent of total particulate radioactivity found in each particulate fraction and percent of total recovered activity (includes 100,000 x g supernatant activity) in each particulate fraction. Some of the label in the particulate fractions could be contamination because of the single pellet wash; hence, not too much emphasis should be put on the data.

Emery (96) also studied distribution of corticosterone binding in particulate fractions. He expressed

TABLE 10.--Distribution of corticosterone-H^3 among three subcellular, centrifugally produced particulate fractions.

Fraction	% of total label recovered in particulate fractions			% of total label recovered (includes supernatant)		
	$\bar{x}$	±	S.E.	$\bar{x}$	±	S.E.
600 x g	41.1		0.74	2.02		0.18
30,000 x g	27.4		1.77	1.35		0.17
100,000 x g	31.1		1.90	1.50		0.04

his binding as percent of added activity and found a range of 3.7-6.0% bound in the nuclear fraction (1,000 x g pellet) from rat mammary homogenate. The 30,000 x g pellet from that homogenate contained 0.9 to 16.6% of the added steroid. The 30,000 x g precipitate from bovine mammary tissue bound 6.9 to 7.1% of added steroid. Emery used 10^{-6}M corticosterone vs 2.5 X 10^{-8}M used in this experiment and the 40-fold higher concentration he used might be responsible for much of the difference between the two sets of data.

Further study of particulate steroid-binding was done under different conditions. A homogenate was prepared in TMK from oxytocin-penicillin-treated frozen lactating-bovine mammary tissue. It showed much more binding activity than the previous homogenates prepared in 0.1M Tris buffer. At 8.3 X 10^{-9}M corticosterone concentration the 750 x g, 27,000 x g and 100,000 x g fractions bound 9.3, 3.9, and 0.3% of the recovered label, respectively. The percents of the total steroid bound in the total particulate fractions were 69.2, 28.7, and 2.1 for each of the three fractions. Those values are quite different from the 41.1, 27.4, and 31.1% found previously. The apparent differences are the treatment

of the tissue before freezing, the molarity and the pH of the buffer, better pellet rinsing and the use of less than one half the previously used corticosterone concentration.

An experiment was performed to compare total particulate steroid binding at four different corticosterone concentrations. Aliquots of homogenate were incubated at 0.83, 1.66, 3.32, and 4.98 X 10^{-9}M corticosterone, centrifuged 27,000 x g, washed four times, and the pellets, washes, and supernatants counted. The washed 27,000 x g precipitate from the 0.83 X 10^{-9}M corticosterone incubation contained 10% of the total recovered label. The 10% represented the most efficient (highest percent of recovered label) binding among the four steroid concentrations and indicated that higher proportions of steroid are bound at lower concentrations.

None of the foregoing experiments considered binding activity which might be present in the 100,000 x g supernatant. This soluble, or cytoplasmic, binding was shown by passage of labeled 30,000 x g supernatant through G—100. From the label in the G-100 macromolecular peak the amount of binding due to the 100,000 x g particulate fraction must be subtracted in order to determine the

soluble binding. Some experiments which could be used to calculate the percentage of particulate binding in the 30,000 x g supernatant had been performed. One experiment, using a homogenate incubated with 2.5×10^{-8}M corticosterone had shown only 1.5 to 1.7% of the label in the 30,000 x g supernatant to be due to the 100,000 x g pellet. In another experiment the 100,000 x g fraction from homogenate incubated at 8.3×10^{-9}M corticosterone contained only 0.3% of the 30,000 x g supernatant label. That represents one-fifth the binding activity of the first experiment at one-fifth the steroid concentration used in the first experiment. This observation was applied to the measurement of the binding protein in a 30,000 x g supernatant of a homogenate which had been incubated with 1.8×10^{-10}M corticosterone and passed through G-100. The macromolecular peak from the G-100 had 7.9% of the label recovered from the column. The work with the 100,000 x g pellet had shown that only a small proportion, less than 1.7% of that 7.9% from the G-100 would be due to 100,000 x g sedimentable material. Therefore the 7.9% of the 30,000 x g supernatant label found in the G-100 exclusion peak apparently represented

considerable binding in the "cytoplasmic" (100,000 x g supernatant) portion of the mammary cell.

Purification of Solubilized Binding Protein

Studies which are designed to determine the physical characteristics of a binding protein require that the protein be isolated in pure form. The purpose of this study was to show the existence of a glucocorticoid binding protein in mammary gland and to study some of its binding kinetics. For such purposes a totally purified preparation is not required and the purification attempted and accomplished during this study was incomplete. The successful fractionation, purification, steps reported here could serve as the initial procedure for a total purification. Fractionation was performed on soluble cytoplasmic proteins or NaOH-solubilized particulate protein using ammonium sulfate, Sephadex G-100, calcium phosphate gel, and DEAE-cellulose.

Ammonium sulfate

One of those fractionation methods, ammonium sulfate precipitation, is generally considered a gentle

method of protein separation, and is frequently the first step in enzyme purifications. As such it was the initial method tested in this study.

Ammonium sulfate precipitation could conceivably dissociate steroid from binding protein. Such an effect would negate any radioactivity measurements of binding made on material labeled before ammonium sulfate precipitation. Dissociation, although not exactly of that nature, was observed during pellet washing and molecular seive chromatography and was discussed in the first portions of this Results and Discussion section. Although such dissociation had been observed the work with ammonium sulfate gave some insight to a tenacious binding being maintained during those ammonium sulfate precipitations. The earliest of the ammonium sulfate fractionations indicated that steroid would co-precipitate with the protein and when resuspended would appear as bound steroid.

An experiment to study the amount of bound steroid surviving ammonium sulfate precipitation and resuspension started with a 30,000 x g fraction from rat mammary tissue. It was incubated with 1.66 X 10^{-8}M corticosterone-H^3, recentrifuged, resuspended in 0.05 N NaOH and made to 50% saturated ammonium sulfate. The ammonium sulfate

precipitate was resuspended in 0.05 N NaOH and passed through G-100 to determine radioactivity in the protein peak. That protein peak contained thirty-four percent of the steroid which was bound before ammonium sulfate precipitation. That much steroid had remained bound through the precipitations and resuspensions. When the same resuspended ammonium sulfate precipitate was reincubated with 8.3×10^{-9}M corticosterone-H^3, the amount of tritium associated with the protein fraction from G-100 did not greatly increase. When the 50% saturated ammonium sulfate supernatant was passed through G-100 only 13.5% of the steroid bound before the ammonium sulfate treatment was in the protein peak. Therefore, 52.5% of the originally bound steroid was lost by the ammonium sulfate precipitation.

Possible explanations for the results are available. Precipitation could have released some of the steroid which would then be diluted and lost in the supernatant, however, only 13.5% of the steroid in the 50% saturated ammonium sulfate supernatant appeared to be bound. Therefore, there was either very little binding protein in the supernatant or it was unable to bind more than 13.5% of the available steroid in the presence of

50% saturated ammonium sulfate. Although ammonium sulfate was removed from the precipitated protein by G-100, the available steroid was also being removed so the renaturated protein, if it did become renatured, would not have steroid available to bind.

Permanent denaturation by the ammonium sulfate precipitation is another explanation. Reincubation of the precipitated material did not greatly increase the amount of steroid bound. Permanent denaturation could give such a result but another alternative is that not all of the binding protein was precipitated by addition of ammonium sulfate, to 50% saturation. Later work, with bovine mammary, indicated that more binding activity precipitated between 50 and 80% saturated ammonium sulfate than 0-50%.

One other cause for concern would be that steroid will precipitate with protein because association with protein would provide a more favorable environment for the hydrophobic steroid than would the highly polar ammonium sulfate solution. To determine if such was the case 10 ml of a 1 mg per ml bovine serum albumin (BSA) solution was heated 25 min at 60°C to denature the protein and destroy its steroid-binding ability. It was

incubated with 1.7 X 10^{-10}M corticosterone and precipitated by making the solution to 80% saturated ammonium sulfate. Forty-nine percent of the steroid precipitated with the BSA although even undenatured BSA would not normally bind any significant amount of steroid at such a low steroid concentration. Apparently unbound steroid will precipitate with protein.

Ammonium sulfate precipitation has been used to study renal aldosterone-binding proteins (108). The nuclear fraction from kidneys of rats injected with aldosterone was extracted and the protein in the extract was precipitated by 50% ammonium sulfate and then resuspended. After a second ammonium sulfate precipitation only 78% of the label precipitated the first time was reprecipitated by that second ammonium sulfate precipitation. The second precipitate was resuspended and passed through Sephadex G-50. Only 50% of the steroid was bound to protein. The authors attributed the loss of "bound" steroid to dissociation on the Sephadex but the data seems to indicate noticeable precipitation of free steroid by 50% ammonium sulfate. The results of the aldosterone work of Herman et al. (108) and the coprecipitation of

steroid with BSA shown in this study necessitates caution in relating ammonium sulfate precipitated-steroid and binding.

Purification by ammonium sulfate fractionation was initially done with a NaOH extract of the 30,000 x g fraction. The NaOH extract was dialyzed against water to reduce the pH and then the ammonium sulfate fractions were prepared. Protein concentration was determined by 260/280 absorbance in the supernatants of each fraction and radioactivity was determined in the ammonium sulfate precipitates. By those admittedly crude means, the highest specific activities were found in the 40-50, 50-60, and 60-80% saturated ammonium sulfate fractions. Faulty protein determinations precluded judgment on the actual extent of the binding but the specific activities of the 40-80% fractions were at least 2.8 times those of the 0-40% fractions and 2.2 times the specific activity of the supernatant from which they came.

Ammonium sulfate also precipitated a binding protein from the 30,000 x g supernatant of a 750 x g supernatant which had been incubated with 2.7×10^{-10} M corticosterone-H^3. The 30,000 x g supernatant was dialyzed against 2 mM K_2HPO_4 (pH 7.6) containing charcoal

and from the dialyzed material 0-30, 30-40, 40-50, 50-60, and 60-80% saturated ammonium sulfate fractions were prepared with radioactivity determined in the precipitates and 260/280 absorbance protein determinations done on the supernatants. Due to protein determination difficulties the 0-30 and 40-50% fractions had infinite cpm/mg protein. Disregarding those fractions most of the binding protein appeared to be in the 60-80% fraction.

Another 750 x g supernatant was fractionated with saturated ammonium sulfate added to 0-30, 30-40, 50-60, and 60-80%. The ammonium sulfate precipitates were resuspended with 0.5 N NaOH in TMK, incubated with 3.3 X 10^{-10}M corticosterone-H^3, then dialyzed 10 hours against TMK and charcoal. Aliquots of the dialysate were counted and 260/280 absorbance and Lowry protein determinations performed. Both protein determination methods showed the highest specific activity to be in the 60-80% fraction though it was only 26% the specific activity found in the previously mentioned 60-80% saturated ammonium sulfate fraction of a 30,000 x g supernatant. The discrepancies can probably be attributed to inconsistency of the protein determinations, differences in the dialyses, or whether

the material was precipitated before or after incubation with steroid.

Another aliquot of the 30,000 x g supernatant was made to 0-50 and 50-80% with saturated ammonium sulfate and the resulting precipitates resuspended, dialyzed to remove ammonium sulfate and frozen. These fractions were later thawed, incubated with corticosterone-H^3 and then dialyzed against TMK and charcoal as in the previous experiment. The dialysates contained 819-967 cpm/mg protein in the 50-80% fraction and only 97 to 119 cpm/mg in the 0-50% fraction. In later work, equilibrium dialysis of the 0-50 and 50-80% saturated ammonium sulfate fractions at 1.66×10^{-11} M steroid concentration showed unequivocally that the 50-80% saturated ammonium sulfate fraction contained most of the binding protein recoverable from the 30,000 x g supernatant. The 50-80% fraction after equilibrium dialysis bound seven times more label than the 0-50% fraction from an equivalent amount of tissue.

In order to more accurately determine the ammonium sulfate concentration required for precipitation of the binding protein, 50-60, 60-70, and 70-80% fractions were prepared from a 30,000 x g supernatant. After removal of ammonium sulfate by dialysis against TMK, the ability of

the fractions to bind steroid was measured by equilibrium dialysis at 1.66 X 10^{-11}M corticosterone-H^3. The three ammonium sulfate fractions listed above had 79, 312, and 424 cpm/mg protein, respectively, again indicating highest binding activity in the precipitates from the higher ammonium sulfate fractions. On the basis of all the foregoing ammonium sulfate precipitations, most of the studies of competition and binding affinities were done on the 60-80% saturated ammonium sulfate fraction from 30,000 x g supernatants.

Calcium phosphate gel

Calcium phosphate adsorption is another method of separating proteins. Proteins can be either adsorbed onto calcium phosphate gel and then be selectively released from the gel, or contaminating proteins can be adsorbed leaving the protein of interest in solution. The latter method was the first approach used in this study. Increasing concentrations of gel were added to NaOH-solubilized particulate proteins and the 30,000 x g supernatant in an attempt to remove "contaminating" proteins from solution. The NaOH extract used was prepared

from the 30,000 x g pellet from a homogenate incubated with 3.2 X 10^{-10}M corticosterone-H^3. The NaOH extract was dialyzed against water to a pH between 6.5 and 7.0 and any dialyzed material that precipitated was removed by centrifugation. To 8.4 ml of that dialyzed solution, which represented about 1.5 g tissue, 0.3% (dry weight/volume) calcium phosphate gel was added. Material not adsorbed to that 0.3% gel was subjected to 0.58% gel and the remainder from that subjected to 1.1%. Each sample was centrifuged out of suspension and then extracted twice with 1 ml 0.1 M K_2HPO_4. The concentration of proteins extracted from the gels was determined by 260/280 adsorbance. The first two additions of gel apparently removed "contaminating" proteins, i.e., those proteins less able to bind and hold corticosterone during the extractions (Table 11). The K_2HPO_4 extracts from that third quantity of gel showed the highest specific activity indicating that the protein in those extracts tenaciously held corticosterone through the adsorption to the gel.

The same type of procedure was used to fractionate a 30,000 x g supernatant which was prepared from a 750 x g supernatant incubated with 3 X 10^{-10}M corticosterone-H^3.

TABLE 11.--Recovery of protein and radioactivity from increasing concentrations of calcium phosphate gel added to the dialyzed NaOH extract of a 30,000 x g particulate fraction previously incubated with corticosterone-H^3.

% gel (dry wt/vol)	extract	mg protein	CPM	CPM/mg protein
0.30	1	1.90	108	56.8
	2	1.20	48	40.0
0.58	1	2.30	148	64.3
	2	1.95	86	44.1
1.10	1	1.05	156	148.6
	2	1.20	128	106.7

The 30,000 x g supernatant was dialyzed against 2mM K_2HPO_4 (pH 7.6) containing charcoal. After dialysis 0.36, 0.68, 0.96, 1.22, and 1.45% calcium phosphate was successively added to 7.1 ml of the dialyzed supernatant. Each fraction was then extracted twice with 1 ml 0.5M K_2HPO_4 to elute adsorbed protein. Those protein concentrations were determined by 260/280 adsorbance and used to calculate the specific activities shown in Table 12. As in the calcium phosphate fractionation of the NaOH extracts, nonbinding protein was removed by the first additions of gel while the gels in concentrations over about 1% adsorbed steroid-binding protein.

The alternative approach to calcium phosphate fractionation was used to fractionate an ammonium sulfate fraction. A relatively large quantity of gel was added to the ammonium sulfate fraction and then the protein was selectively eluted from the gel. For that procedure a dialyzed 70-80% saturated ammonium sulfate fraction from a 30,000 x g supernatant was used. One ml of that fraction contained about 1.2 mg protein. To 1 ml was added 0.037 g (dry weight) calcium phosphate gel in 1 ml. After incubation the gel was rinsed three times with 2 ml of 1 mM K_2HPO_4 (pH 7.4) and successively extracted three

TABLE 12.--Recovery of radioactivity per mg protein from increasing concentration of calcium phosphate gel added to labeled 30,000 x g supernatant incubated with corticosterone-H^3.

% gel (dry wt/volume)	Extract 1	Extract 2
	cpm/mg protein	
0.36	31.0	20.4
0.68	36.9	39.8
0.96	215.4	102.0
1.22	447.8	257.6
1.45	377.3	302.6

times each with 2 ml of 50 mM K_2HPO_4 (pH 7.4) and 2 ml of 1 M K_2HPO_4. Portions of each extract were subjected to equilibrium dialysis at 1.66 X 10^{-11}M corticosterone-H^3 and aliquots counted. Using the 260/280 absorbance protein determinations on the extracts before equilibrium dialysis, the cpm/mg protein for the material not absorbing to the gel and the 50 mM and 1 M extracts were 130, 183, and 110, respectively. Apparently there was little further fractionation of the 70-80% saturated ammonium sulfate fraction by the calcium phosphate gel.

Disc gel electrophoresis was used to monitor the degree of protein separation by calcium phosphate gel fractionation. A dialyzed 60-80% saturated ammonium sulfate fraction was fractionated on calcium phosphate in the manner previously described for the 70-80% fraction. Equilibrium dialysis of the gel supernatant and extracts showed 15, 229, and 35 cpm bound per ml. With that distribution, if the binding protein separates from other proteins into a single band upon electrophoresis and equal volumes are electrophoresed, the 50 mM K_2HPO_4 extract (229 cpm/ml) should show a band 15 times as dense as an equivalent band in the supernatant (15 cpm/ml) and 6 times as dense as the equivalent band from the 1 M

extract (35 cpm/ml). Only one band corresponded to that type of binding activity distribution. The material not adsorbing to the phosphate gel showed 11 bands and the 50 mM and 1 M K_2HPO_4 extracts of the gel showed 12 bands. The bands were then compared to find the one corresponding to the concentration of binding activity placed on the electrophoresis gels. The 60-80% saturated ammonium sulfate fraction, put on the gel in small quantity because of its high protein concentration, had a very faint band at R_{dye} (R_d) 0.63. The material not absorbing to the gel had a band of R_d 0.57, the 50 mM extract a heavier band at R_d 0.60 and the 1 M extract had a light band of R_d 0.59. The 60-80% fraction also had a faint band at R_d 0.54 but the calcium phosphate fractions had no bands closer to the 0.57-0.60 bands than R_d 0.47 and 0.66. The band seen between R_d 0.57 and 0.60 was therefore probably the same protein in each of the three fractions and the only one corresponding in density with the distribution of the bound steroid.

Sephadex

Sephadex, previously used to remove free from bound steroid, was also tested as a fractionation method

after ammonium sulfate, by passing 1.0 ml of the 70-80% saturated ammonium sulfate fraction through a 1.5 X 25 cm Sephadex G-100 column eluted with water. Timed fractions were collected and six protein peaks of three fractions each were obtained. Each protein fraction was subjected to equilibrium dialysis against TMK with 1.66 X 10^{-11}M corticosterone-H^3. After equilibrium dialysis specific activities, along with the 280 nm absorbance profile of the Sephadex fractions, are shown in Figure 3.

The binding protein apparently eluted from the column with the largest protein fractions. The highest specific activity among the fractions never reached the 424 cpm/mg protein found in the original 70-80% saturated ammonium sulfate fraction and although there appeared to be some separation of proteins the loss of specific activity negated any gain from their separation on Sephadex.

DEAE-cellulose

DEAE-cellulose ion exchange chromatography was also used to fractionate the 60-80% saturated ammonium sulfate fraction. One ml of the 60-80% fraction was put

Fig. 3.--Sephadex G-100 fractionation of 70-80 ammonium sulfate fraction. Absorbance (280 nm) profile of 1.0 ml 70-80% saturated ammonium sulfate fraction passed through a 1.5 X 25 cm G-100 column equilibrated and eluted with water. Six combined fractions then equilibrium dialyzed against 1.66 X 10^{-11}M corticosterone-H^3 in TMK and cpm bound per mg protein calculated after dialysis.

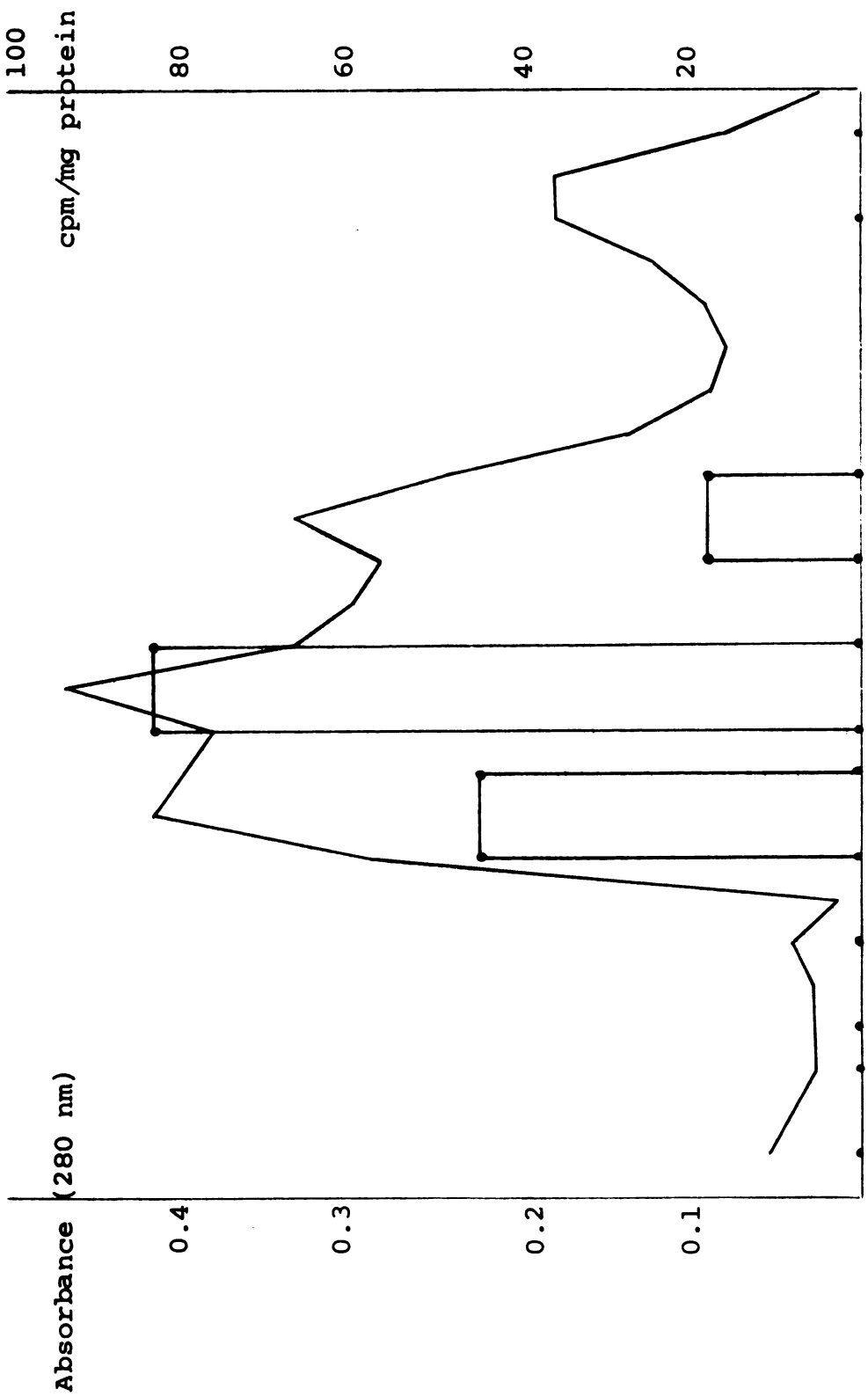
Absorbance (280 nm)
0.4
0.3
0.2
0.1
cpm/mg prptein
100
80
60
40
20
1
2
3
4
5
6
Fractions

Figure 3

on a 10 ml DEAE-cellulose column equilibrated as described in the Material and Methods section. The fractions were then eluted with a gradient from 1 mM K_2HPO_4 (pH 7.4) to 1 M K_2HPO_4. Timed fractions were collected and combined to form the six fractions shown in Figure 4. These fractions were subjected to equilibrium dialysis against TMK with 1.66 X 10^{-11} M corticosterone-H^3. After equilibrium dialysis specific activities were calculated using Lowry protein determinations for protein concentrations. The specific activities and 280 nm absorbances are shown in Figure 4.

Separation of the binding protein was better on the DEAE-cellulose than on the Sephadex since there was less overlap between fractions and much higher specific activity was found by DEAE separation. The highest specific activity found after DEAE separation represented 2.3 times that of the 60-80% saturated ammonium sulfate fraction from which it came.

Immunological comparisons

Glucocorticoid-binding material had been found in both NaOH extracts of particulate fractions and in the

Fig. 4.--DEAE-cellulose fractionation of 60-80 ammonium sulfate fraction. Absorbance (280 nm) profile 1.0 ml 60-80% saturated ammonium sulfate fraction eluted from 10 ml DEAE-cellulose by a gradient of 1 mM pH 7.4 K_2HPO_4 to 1 M K_2HPO_4. Six combined fractions then equilibrium dialyzed against 1.66 X 10^{-11}M corticosterone-H^3 in TMK and cpm bound per mg protein calculated after dialysis.

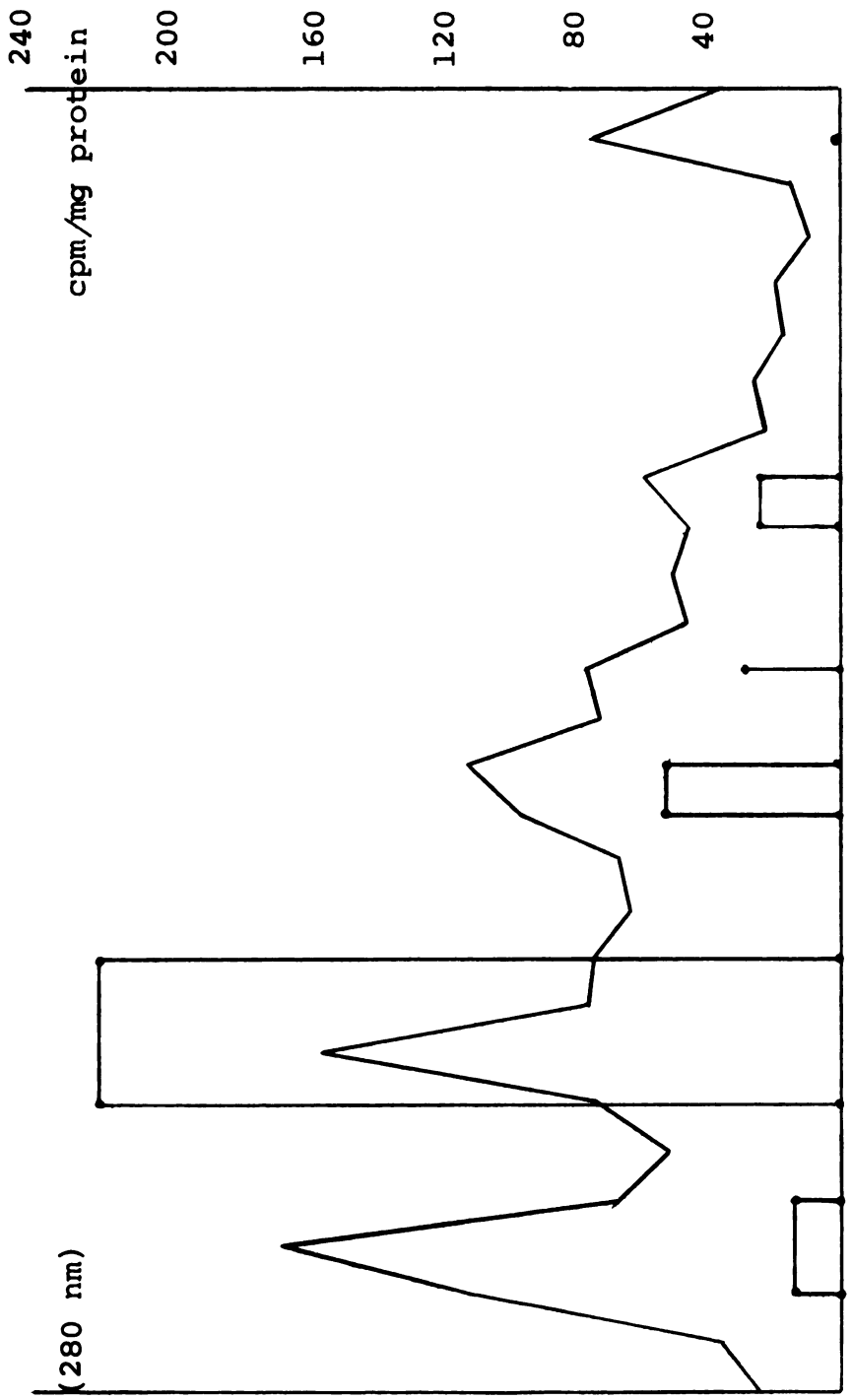
Absorbance (280 nm)
0.6
0.5
0.4
0.3
0.2
0.1
cpm/mg protein
240
200
160
120
80
40
1
2
3
4
5
6
Fractions

Figure 4

ammonium sulfate fractions from 30,000 x g supernatant. The binding materials could conceivably be the same protein due to adsorption of soluble, cytoplasmic, protein to the particulate fractions. The possibility of such cross contamination was examined by immunological techniques. Rabbits were immunized against the 60-80% saturated ammonium sulfate fraction and their serum later reacted against both the 60-80% fraction and the 30,000 x g NaOH extract. Though the precipitin bands on the Ouchterlony plates were broad and smeared, much of the reaction area was similar and some identity between precipitin bands could be assumed. At least one precipitin band present in the 60-80 fraction was absent in the NaOH extract, otherwise the reactions were indistinguishable. The similar reactions indicate the possibility that "particulate" binding was due to adsorption of soluble binding protein to the particulate fractions.

Binding Characteristics

Competition Studies

Binding of a steroid hormone to a protein is relevant only if some degree of specificity for that

hormone can be demonstrated. Competition experiments are therefore very important to studies of a particular binding protein. Such experiments were performed in a number of ways on the glucocorticoid binding protein under study.

The competition for corticosterone binding sites by various steroids was very difficult to study during the initial portions of this study. In the first attempts, homogenates were incubated with 1.7×10^{-8}M corticosterone-H^3 and very high (more than 1000-fold higher) concentrations of progesterone, hydrocortisone, or corticosterone and the three particulate fractions produced from those homogenates were assayed for incorporated label. The results are shown in Table 13 as percentage of total recovered label found in each of the particulate fractions. Neither progesterone nor hydrocortisone would compete with corticosterone, however, unlabeled corticosterone also did not dilute the amount of label bound. The results led to the concept of "infinite," or unsaturable, steroid binding.

Another experiment was performed with 2 ml of 1:4 diluted homogenate incubated with a constant amount, 4.9×10^{-9}M, of corticosterone-H^3 and concentrations of corticosterone-C^{14} increasing from zero to 9.9×10^{-7}M.

TABLE 13.--Incorporation of corticosterone-H^3 into particulate fractions and "competition" by progesterone, hydrocortisone and unlabeled corticosterone.

Fraction	Experiment						
	1		2		3		
	C*	P	C	HC	C	HC	C+C
600 x g	2.1**	2.1	1.7	2.4	1.8	2.2	2.0
30,000 x g	1.6	1.7	0.9	1.1	1.3	1.4	1.4
100,000 x g	1.5	1.8	1.5	1.5	1.4	1.4	1.5

*C - corticosterone-H^3, P - progesterone, HC - hydrocortisone, C+C - unlabeled corticosterone plus corticosterone-H^3.

**% of total recovered label.

Double isotope counting allowed calculation of the total corticosterone bound. The μμ moles corticosterone recovered in the washed 750 x g and 27,000 x g fractions are shown in Figure 5. The total μμ moles steroid recovered are indicative of the increasing steroid added and the steadily increasing uptake of label with increasing initial steroid concentration is obvious.

The assumption was then made that there were at least two types of binding sites with different affinities for corticosterone. Therefore, the only way to show good competition between labeled and unlabeled corticosterone would be to work in a narrow, low-steroid concentration range where the high affinity, "finite," "saturable" binding sites give a curvalinear uptake of steroid rather than at higher steroid concentrations where the apparently unsaturable, "infinite" binding is linear and tends to mask competition. Before competition could be shown between corticosterone-H^3 and other steroids it was necessary to demonstrate competition between corticosterone-H^3 and its nonlabeled analog.

Two experiments using low steroid concentrations and dialysis against charcoal were done to show competition between corticosterone and corticosterone-H^3. In

Fig. 5.--Corticosterone bound in two particulate cell fractions incubated with four levels of corticosterone. Two-tenths gram homogenized tissue in 2 ml TMK incubated with corticosterone ($-H^3$ and $-C^{14}$) 60 min at room temperature. Particulate fractions washed three times each and corticosterone extracted with ethanol for counting.

•—• 750 x g fraction

o—o 27,000 x g fraction

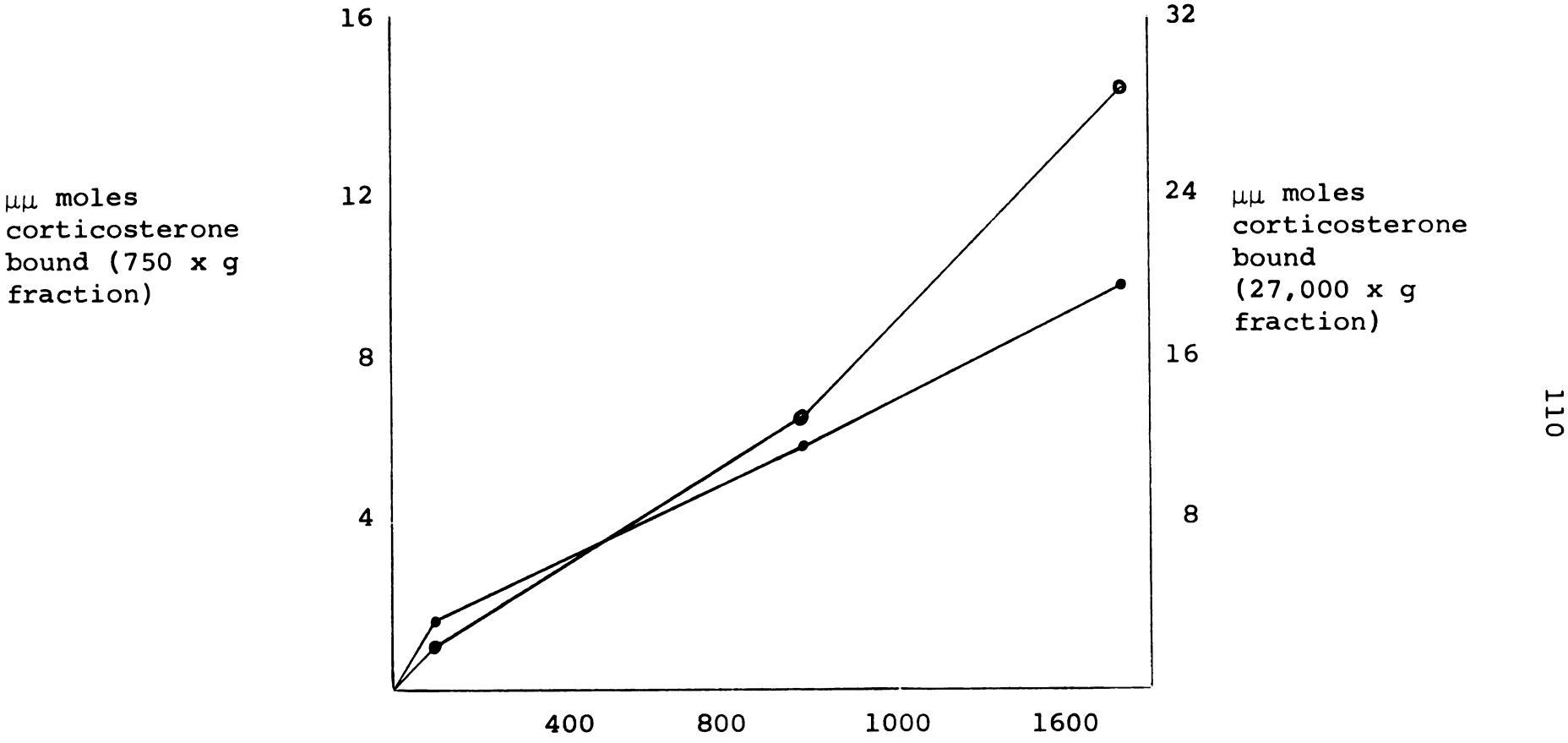
16
12
8
4
32
24
16
8
μμ moles corticosterone bound (750 x g fraction)
μμ moles corticosterone bound (27,000 x g fraction)
400
800
1000
1600
Total μμ moles corticosterone recovered

Figure 5

the first experiment three 2.5 cm dialysis sacs, each containing 5 ml of a 1:4 dilution of a 750 x g supernatant or TMK, were suspended in 300 ml TMK containing 1 to 2 g activated charcoal in suspension. Conditions for the second experiment were the same except that each sac contained 10 ml of diluted 750 x g supernatant. Each sac, within the same experiment, contained equal concentrations of corticosterone-H^3. One sac contained only TMK while two sacs, one of which had unlabeled corticosterone added, contained the diluted 750 x g supernatant. The levels of labeled and unlabeled corticosterone added initially and steroid remaining in the sac after 8 hours dialysis are shown in Table 14.

During the experiment samples had been removed from the sacs during the first hour of dialysis in order to check the rate of dialysis. Those samples showed that equal percentages of the initial radioactive label were being dialyzed out of each sac although the "high steroid" sac had much more steroid per unit of label. The sac with the highest steroid concentration lost 5×10^{-11} moles corticosterone per hour averaged over 8 hours. Because equal percentages of label were dialyzed out of the sacs the percentage of label left in the sac without

TABLE 14.--Loss of corticosterone from TMK and 750 x g supernatant dialyzed against TMK and charcoal.

	TMK	Supernatant 1	Supernatant 2
Experiment 1			
0 hr steroid concentration (X 10^{-10}M)	33	33	899
8 hr steroid concentration (X 10^{-10}M)	2.24	3.57	87.76
8 hr/0 hr X 100	6.8	10.8	9.7
8 hr unbound steroid (X 10^{-10}M)	2.24	2.24	60.66
8 hr bound steroid (X 10^{-10}M)	0	1.33	27.10
Experiment 2			
0 hr steroid concentration (X 10^{-10}M)	8.3	8.3	441
8 hr steroid concentration (X 10^{-10}M)	0.43	0.91	32.44
8 hr/0 hr X 100	5.2	10.9	7.3
8 hr unbound steroid (X 10^{-10}M)	0.43	0.43	22.94
8 hr bound steroid (X 10^{-10}M)	0	0.48	9.50

750 x g supernatant after 8 hours dialysis was indicative of the unbound steroid in the other two sacs. Therefore an amount of steroid proportional to that in the TMK (no 750 x g supernatant) sac could be subtracted from the residual steroid in the other two sacs in order to calculate bound and unbound steroid in those sacs. If the steroid bound increased proportionate to steroid concentration all four sacs containing binding protein would retain the same proportion of initial steroid; i.e., all four 8 hr/0 hr ratios would be identical. The fact that the two sacs with the highest steroid concentrations both retained a lower percentage of initial steroid showed that binding sites were being saturated (corticosterone molecules were competing with each other for binding sites). Increasing the steroid concentration should, but did not, give steadily decreasing retention of steroid proportional to initial steroid concentration. The proportions of label retained at increasing steroid levels were 10.9, 10.8, 7.3, and 9.7%. There is no apparent reason for such a discrepancy.

The percentage of initial corticosteron-H^3 remaining after 8 hours dialysis clearly showed competition

between corticosterone and corticosterone-H^3. However, if available binding sites had been saturated with steroid at the low concentrations there should have been 27- and 53-fold decreases in label retained in Experiments 1 and 2, respectively. The differences were not nearly that large indicating either failure to saturate one type of binding site or saturation of one type of site and partial saturation of other sites having lower affinity for corticosterone.

Successful demonstration of competition, though not of the magnitude expected, by dialysis against charcoal led to a dual label competition experiment with hydrocortisone, progesterone and estradiol. Those hormones, each labeled with C^{14}, were added to supernatant of a 750 x g centrifugation. Corticosterone-H^3 was then added and the mixtures dialyzed against TMK and charcoal. After dialysis aliquots were counted by double label techniques and total steroid concentration calculated (Table 15).

If corticosterone competed equally with any of the other steroids it should have displaced one half of that steroid from its binding sites at equal steroid concentrations and C^{14} and H^3 should be dialyzed out at

TABLE 15.--Loss of corticosterone-H^3, hydrocortisone, progesterone and estradiol from a 750 x g supernatant dialyzed against charcoal suspended in TMK.

	Sac 1		Sac 2		Sac 3	
	HC*	C	P	C	E	C
0 hr dialysis X 10^{-10}M steroid	1780	2.683	938	2.388	499	2.241
20 hr dialysis X 10^{-10}M steroid	5	0.016	33	0.018	48	0.042
% of initial	0.3	0.6	3.5	0.8	9.6	1.9

*HC = hydrocortisone, C = corticosterone-H^3, P = progesterone, E = estradiol.

approximately equal rates. If there was no competition, but each steroid was binding to a separate site within the 750 x g supernatant, more H^3 and C^{14} should have been retained than in the sacs where there was competition. Partial competition should have been intermediate. Because of the unequal concentrations of hydrocortisone, progesterone, and estradiol used in this experiment only the effect of these steroids on corticosterone retention was valid for comparative purposes.

Three degrees of competition were observed with hydrocortisone showing the best competition with corticosterone. Therefore hydrocortisone was apparently competing for corticosterone binding sites. Almost as much corticosterone was dialyzed out of the sac containing progesterone as from the sac with hydrocortisone. Therefore progesterone must have been competing although not to the same extent as hydrocortisone. Corticosterone retention was best in the presence of estradiol and although competition between the two steroids cannot be ruled out in this experiment, there apparently was much less competition from estradiol.

Observations of the C^{14} steroids bound showed more estradiol bound than any of the others. The least binding was with hydrocortisone although it was present in the highest concentration. The difference could have been due to tenacity of binding or number of binding sites available to those steroids in the 750 x g supernatant.

The quantitative extent of competition for corticosterone binding sites was determined with the 60-80% saturated ammonium sulfate fraction of the 30,000 x g supernatant using equilibrium dialysis. The steroids used in the competition studies were cholesterol, estradiol, progesterone, hydrocortisone, and corticosterone. They were tested for their ability to compete with corticosterone-H^3 for binding sites.

The equilibrium dialysis was performed with sacs containing 2 ml of diluted (1:9 with TMK) 60-80% saturated ammonium sulfate fraction from fresh lactating bovine mammary. The sacs were placed in test tubes containing buffer and corticosterone (total volume, 100 ml). Corticosterone-H^3 was added to 8.3×10^{-11}M. The quantities of cholesterol, estradiol, progesterone, hydrocortisone and corticosterone added are shown in Table 16. After equilibrium dialysis aliquots of inside and outside

TABLE 16.--Concentrations of various hormones added to compete with 8.3 X 10^{-11}M corticosterone-H^3 in equilibrium dialysis of 60-80% saturated ammonium sulfate fraction of 30,000 x g supernatant.

Tube #	Cholesterol	Estradiol	Progesterone	Hydrocortisone	Corticosterone
	----------X 10^{-11} M----------				
1	0	0	0	0	8
2	259	3670	3180	2755	441
3	2586	7340	6360	5510	4337
4	25860	44040	38160	33060	43298

solutions were counted and the bound to unbound ratios calculated. A graph of the bound to unbound corticosterone ratios vs total steroid concentration is shown for all the steroids in Figure 6. Corticosterone and hydrocortisone both competed with corticosterone-H^3 for binding sites. Progesterone competed to a lesser extent while cholesterol and estradiol either did not compete or competed only at very high concentrations. The competition by progesterone was not unexpected since Tucker et al. (97, 98) had shown progesterone competing with hydrocortisone in their cell culture binding studies.

Binding Constants

The nature of corticosterone binding at various concentrations is elucidated somewhat by calculation of binding constants from data relating the proportion of bound to unbound steroid at each of several steroid concentrations. The bound to unbound ratio is frequently plotted against the concentration of bound steroid. That relationship forms the Scratchard plot described in the Review of Literature section.

Fig. 6.--Competition for corticosterone binding sites by cholesterol, estradiol, progesterone and hydrocortisone. Two ml diluted (1:9 TMK) 60-80% saturated ammonium sulfate fractions dialyzed in 100 ml total volume containing 8.3×10^{-11}M corticosterone-H^3 and different levels of unlabeled steroid.

corticosterone

cholesterol

estradiol

hydrocortisone

progesterone

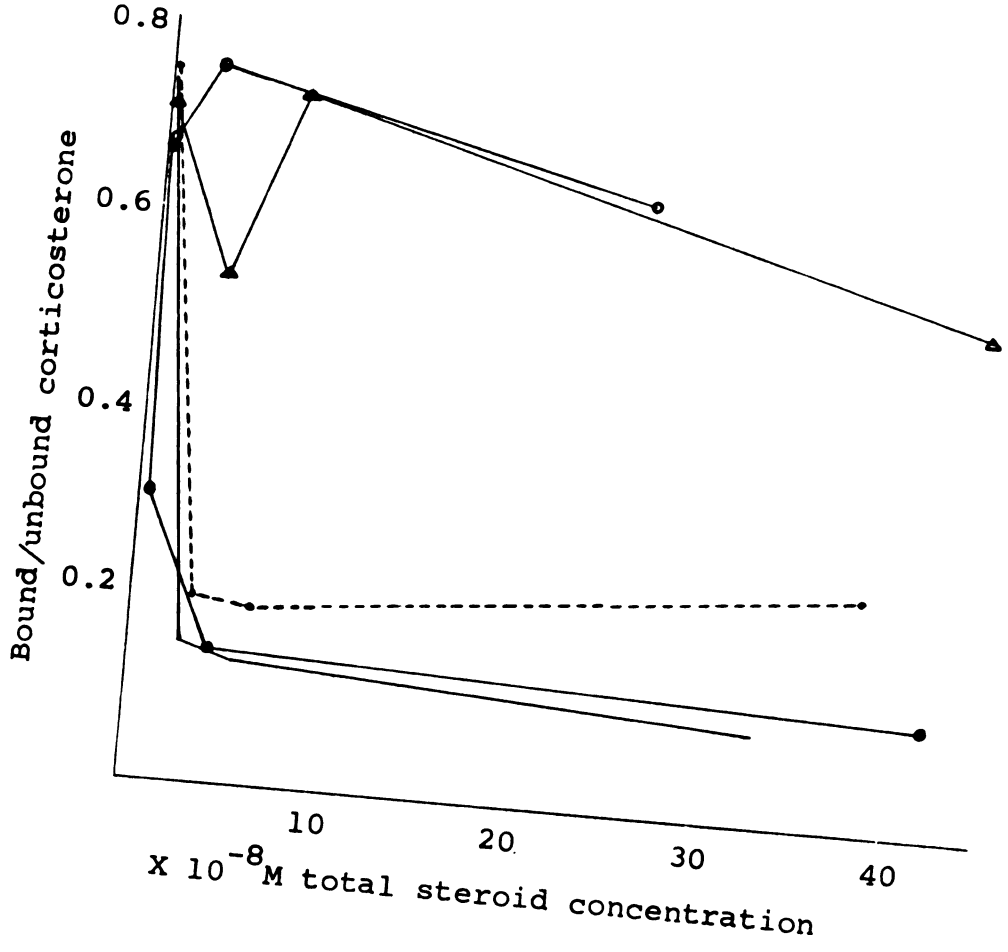
0.8
0.6
0.4
0.2
Bound/unbound corticosterone
10
20
30
40
X 10-8M total steroid concentration

Figure 6

The first data which could be used for a Scatchard plot, although it was not designed for such use, was the data shown in Table 14. That data, although there were only two points in each experiment, was plotted by Scatchard's method and binding constants were determined as shown in Figure 7. The lines can be used only to indicate that one set of points represented higher or lower affinity binding than the other set. The slopes of those lines are the binding constants. They indicate that the data in Experiment 1 was collected in the concentration range which involves low affinity binding rather than high affinity binding. The binding constant in Experiment 2 was more than 10 times lower and therefore indicated higher affinity binding. High affinity binding usually indicates specificity and as such it should therefore be easier to show competition between labeled and unlabeled corticosterone in Experiment 2. That was the case as the discussion of the Table 14 data indicated.

The two experiments shown in Table 14 were performed in similar manner and tempt one to draw a "curve" using all four points on the Figure 7 Scatchard plot. The dashed line in Figure 7 shows such a curve. The last point, the high steroid concentration point, should not

Fig. 7.--Scatchard plot of Table 14 data. Two charcoal-dialysis experiments with the binding constant from each (k_1 and k_2) and the highest affinity binding constant (k_1) from the combined data.

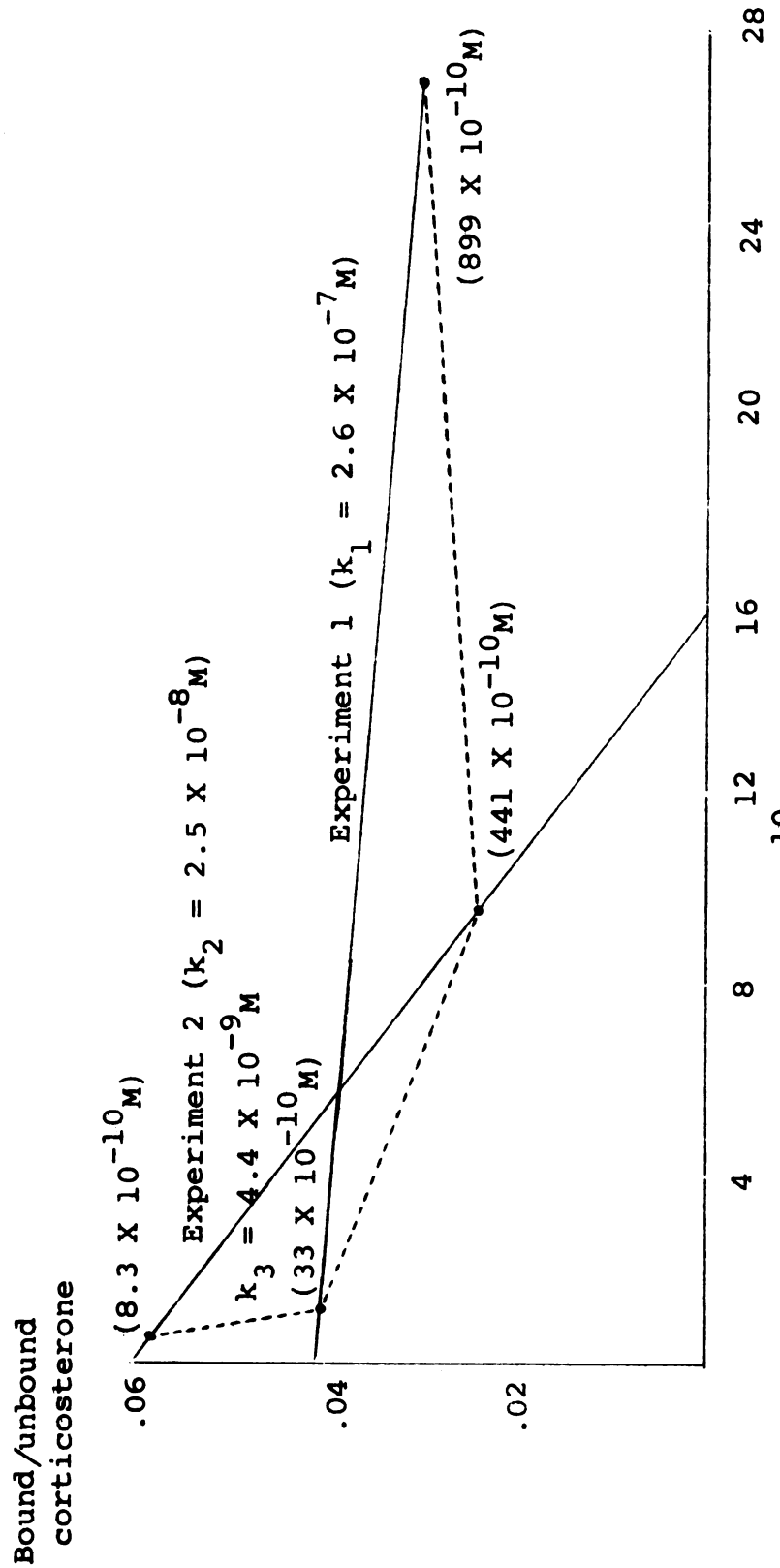
Bound/unbound corticosterone
.06
.04
.02
4
8
12
16
20
24
28
X 10^{-10}M bound corticosterone
(8.3 X 10^{-10}M)
Experiment 2 (k_2 = 2.5 X 10^{-8}M)
k_3 = 4.4 X 10^{-9}M
(33 X 10^{-10}M)
Experiment 1 (k_1 = 2.6 X 10^{-7}M)
(441 X 10^{-10}M)
(899 X 10^{-10}M)

Figure 7

have been higher if the points had been obtained in the same experiment, therefore, the line from Experiment 1 should probably be displaced slightly downward. The initial slope, between the 8.3 and 33 X 10^{-10}M corticosterone concentration points, estimates the high affinity binding constant if all the points had been from one experiment. Over that narrow range of initial corticosterone concentration the binding constant was 4.4 X 10^{-9}M.

A factor which must be considered in discussing the data in Figure 7 is that as dialysis proceeded the steroid concentration decreased inside the sac and dissociation was probably slowly occurring. With slow dissociation the binding observed was probably more dependent on initial steroid concentrations than the 8 hour concentrations and the data was therefore calculated as such. The data in Figure 7 used the 8 hour steroid concentrations to determine bound steroid while the initial steroid was used as "unbound" to calculate bound to unbound ratios. In order to relate the bound to unbound ratios to initial steroid concentration that initial concentration is shown at each point on the figure.

The uncertainties of unbound steroid concentration in the charcoal dialysis method led to use of equilibrium dialysis to find binding constants under equilibrium conditions. The charcoal-dialysis binding constant calculations (data in Table 14) were made using data from 750 x g supernatants; however, binding constants determined by equilibrium dialysis were for the 60-80% saturated ammonium sulfate fraction of the 30,000 x g supernatant. The equilibrium dialysis experiments were performed as described in the Material and Methods section. Two experiments were performed initially, each with tissue from a different cow. The steroid concentrations used, the concentration of bound and free steroid after dialysis, and bound to unbound ratios are shown in Table 17.

When comparing the data in Table 17 with that on Figure 7 the most obvious difference between the charcoal-dialysis experiment shown in Figure 7 and the equilibrium dialysis experiments was the difference in the bound to unbound ratios. The most plausible explanation is that during charcoal-dialysis dissociation was occurring and any dissociation would lower the bound to unbound ratio when initial steroid concentration was used as the unbound steroid concentration. Therefore the binding constants

TABLE 17.--Corticosterone concentrations and respective bound to unbound ratios after equilibrium dialysis of two 60-80% saturated ammonium sulfate fractions from different bovine tissues.

Tissue 1		Tissue 2	
Corticosterone	B:U	Corticosterone	B:U
X 10^{-11} M		X 10^{-11} M	
		1.7	.832
8.3	.667	8.3	.777
		225	.482
441	.301	441	.421
4337	.139		
43298	.112		

calculated from the charcoal-dialysis data were probably for steroid concentration intermediate between the initial and the 8 hour steroid concentrations.

Also obvious in Table 17 is that the second equilibrium dialysis experiment had higher bound to unbound ratios than for equivalent steroid concentrations in Experiment 1. The higher ratios in Experiment 2 may represent differences in protein concentration since the experiments represented two different 60-80% saturated ammonium sulfate fractions prepared from the same weight of different bovine mammary tissues. The ammonium sulfate fraction used in Experiment 2 may have had more total protein, hence, more binding protein, or may have had a higher proportion of binding protein. Since stage of lactation of the cows at slaughter was unknown any change in binding protein quantity with stage of lactation, if it does change, could explain the different ratios, as could the milk, moisture, or connective tissue content of the tissue.

The data from the two experiments was plotted by both the Lineweaver-Burk, or double reciprocal, and Scatchard methods. The double reciprocal plot from Experiment 1 is shown in Figure 8. The x intercept equals

Fig. 8.--Double reciprocal plot of Tissue 1 60-80 ammonium sulfate fraction corticosterone-binding after equilibrium dialysis. Two ml diluted (1:9 TMK) 60-80% saturated ammonium sulfate fraction per dialysis sac, two sacs per 100 ml total volume containing 8.3×10^{-11}M corticosterone-H^3 and different levels of unlabeled corticosterone.

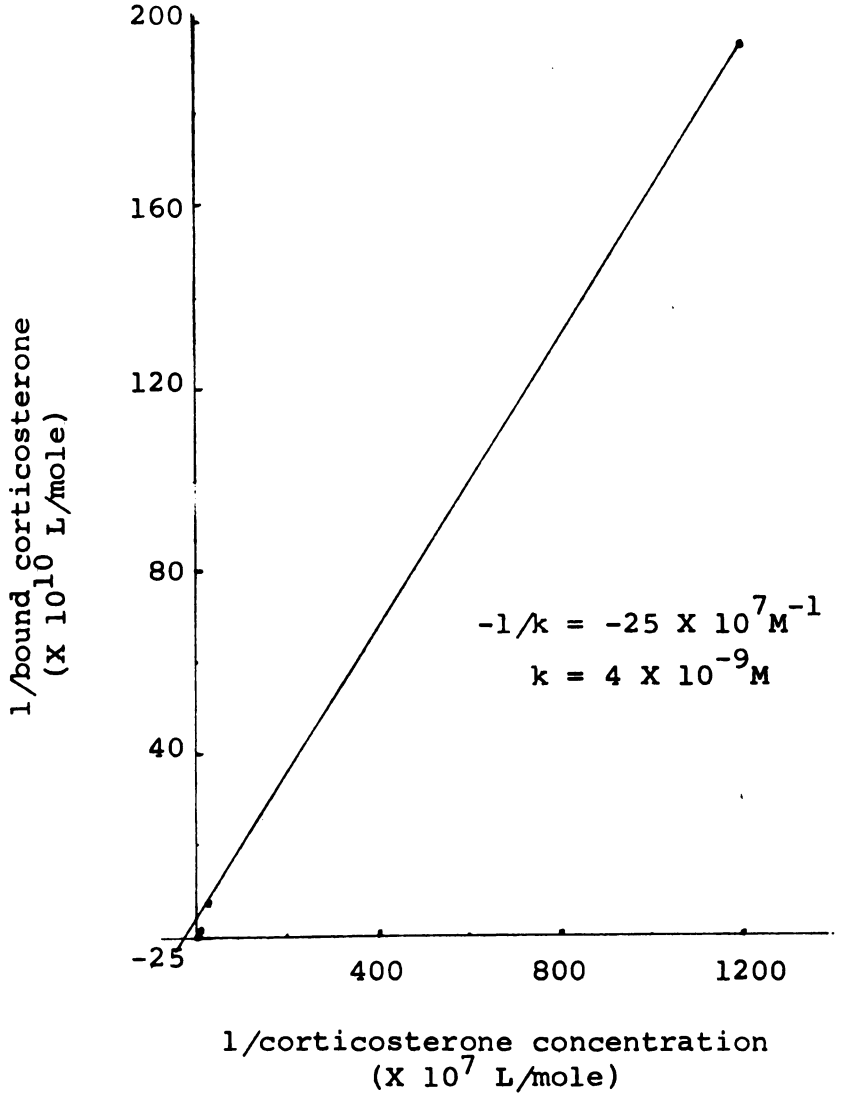
200
160
120
80
40
-25
400
800
1200
1/bound corticosterone
(X 10^{10} L/mole)
1/corticosterone concentration
(X 10^{7} L/mole)
$-1/k = -25 \times 10^{7} M^{-1}$
$k = 4 \times 10^{-9} M$

Figure 8

the negative reciprocal of the binding constant while the y intercept is the reciprocal of the number of binding sites at infinite (1/c=0) steroid concentration.

The problems inherent in the double reciprocal plot are clear in the figure. The high steroid concentrations gave points clustered around the origin and they therefore contributed less to the derived line than did the low concentration points with their wider spread. However, the high affinity binding that is seen at very low steroid concentrations is usually of the most interest and those are the points which are sufficiently separated to allow accuracy in drawing a line through them. In Figure 8 those low concentration points definitely did not create a line which encompassed the points derived from lower affinity binding (high steroid concentration). Over a narrow range of steroid concentration the double reciprocal plot would be valid but over a wide range some of the points have to be ignored. The line in Figure 8 was drawn from only the two low-steroid points. The other two points, clustered at the origin, would make calculation of k, the binding constant, for low affinity binding almost impossible using the scale shown. The line shown in Figure 8 yielded a k of 4×10^{-9}M and since the line

emphasized high affinity binding, the 4 X 10^{-9}M is a high affinity binding constant.

The Scatchard plot of the same data, shown in Figure 9, resolves high and low affinity binding into a more vivid graphical presentation. The low affinity portion of the curve, that part of the curve with the least slope, can be geometrically subtracted from the high affinity portion in order to better determine the high affinity binding constant (71). In Figure 9 the data obtained at 4337 X 10^{-11}M (shown in parenthesis in Figure 9) and 43298 X 10^{-11}M (shown as line 2 in Figure 9) were both considered to represent low affinity binding and each was separately subtracted from the high affinity portion of the curve. Depending on which value was subtracted there was a slight variation in the binding constants calculated. Subtraction of the binding found at 43298 X 10^{-11}M steroid (shown in Figure 7 only as Line 2) from the others yielded a new curve rather than the straight line which the calculations were seeking to produce. By arbitrarily breaking the derived curve into two straight lines (Lines 3 and 4, Figure 9) binding constants of 2.9 and 5.9 X 10^{-9}M were obtained. The 5.9 X 10^{-9}M binding constant was the result of increased low affinity binding because the

Fig. 9.--Scatchard plot of data represented in Figure 8. L1 - L5 = Lines 1-5. Corticosterone concentrations at each point shown in parentheses.

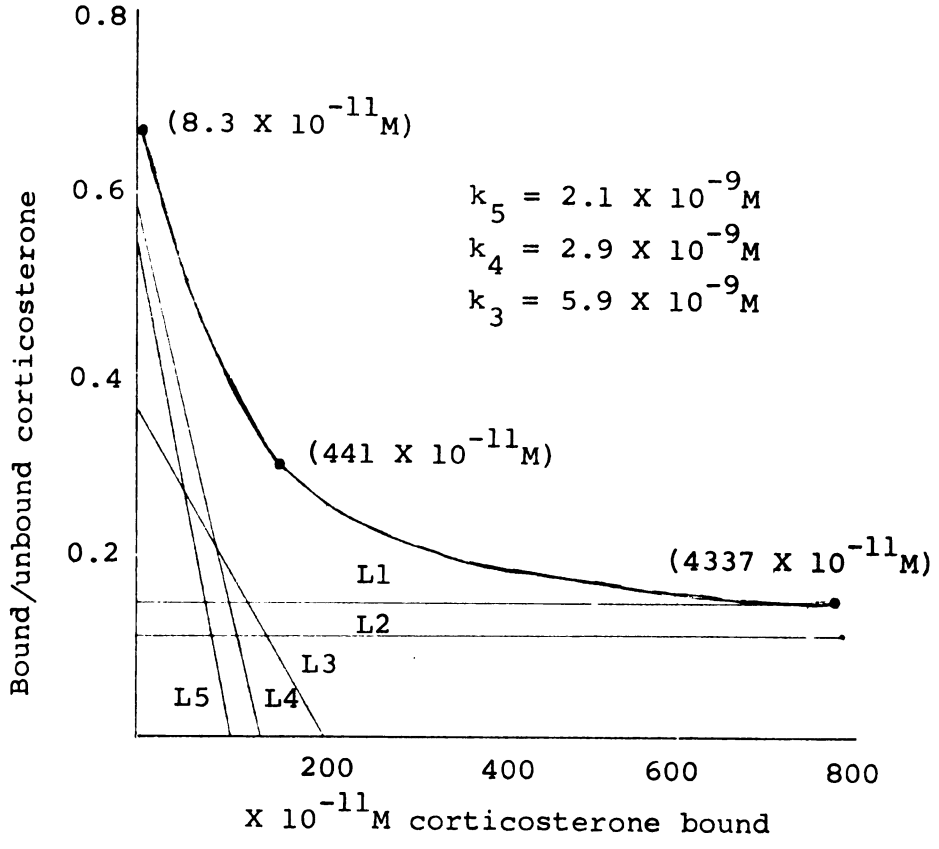
0.8
0.6
0.4
0.2
Bound/unbound corticosterone
(8.3 X 10^{-11}M)
k_5 = 2.1 X 10^{-9}M
k_4 = 2.9 X 10^{-9}M
k_3 = 5.9 X 10^{-9}M
(441 X 10^{-11}M)
(4337 X 10^{-11}M)
L1
L2
L3
L4
L5
200
400
600
800
X 10^{-11}M corticosterone bound

Figure 9

portion of the curve between 441 and 4337 X 10^{-11}M steroid was flattening and therefore had a strong influence on calculation of the binding constant. However, when the binding ratios obtained at 4337 X 10^{-11}M corticosterone were subtracted from the curve they had less influence on the derived curve and the slope of that derived line more closely followed the slope of the initial portion of the original curve. That second derived line gave a binding constant of 2.1 X 10^{-9}M.

As noted in the paragraph above the line between 4337 and 43298 X 10^{-11}M steroid concentration was not shown in Figure 7. The line between those two concentrations became almost horizontal indicating very low binding affinity beyond 4337 X 10^{-11}M corticosterone concentration and conversely, the low steroid concentration data showed progressively smaller binding constants as the steroid concentration decreased. More points must be measured in that area before a really accurate high affinity binding constant can be reliably determined and lower steroid concentrations than those used may yield an even lower binding constant for corticosterone.

In an attempt to better define the high affinity binding constant the second experiment was conducted with

a much narrower range of corticosterone concentration (Table 17). The double reciprocal plot of the data obtained over that narrow range is shown in Figure 10. If in Figure 10, as in Figure 8, emphasis was placed only on the high affinity binding the line would have yielded a k equal to 1 X 10^{-9}M, however, Figure 10 shows the line drawn between the high and low steroid concentration points. Inclusion of the high steroid concentration points was possible because of the narrow range of steroid concentration. Inclusion of the high-steroid, lower affinity points gave a binding constant of 2 X 10^{-9}M. The same data plotted by Scatchard's method is shown in Figure 11. This plot was treated in a couple of ways. Each two successive points were first connected by a straight line, thereby giving three slopes. Those slopes gave k's of 9.6 X 10^{-10}M, 3.5 X 10^{-9}M, and 1.3 X 10^{-8}M. The steepest slope, the highest affinity portion of the data curve, gave the 0.96 X 10^{-9}M which was very close to the 1 X 10^{-9}M determined from the double reciprocal plot when the high affinity binding was emphasized. The least slope may well represent the initial influence of a lower affinity binding which would be seen at high steroid concentrations, while the intermediate slope might

Fig. 10.--Double reciprocal plot of Tissue 2 60-80 ammonium sulfate fraction corticosterone binding after equilibrium dialysis. Two ml diluted (1:9 TMK) 60-80% saturated ammonium sulfate fraction per dialysis sac, one sac per 100 ml total volume containing 1.66 or 8.3 X 10^{-11}M corticosterone-H^3 and two levels of unlabeled corticosterone.

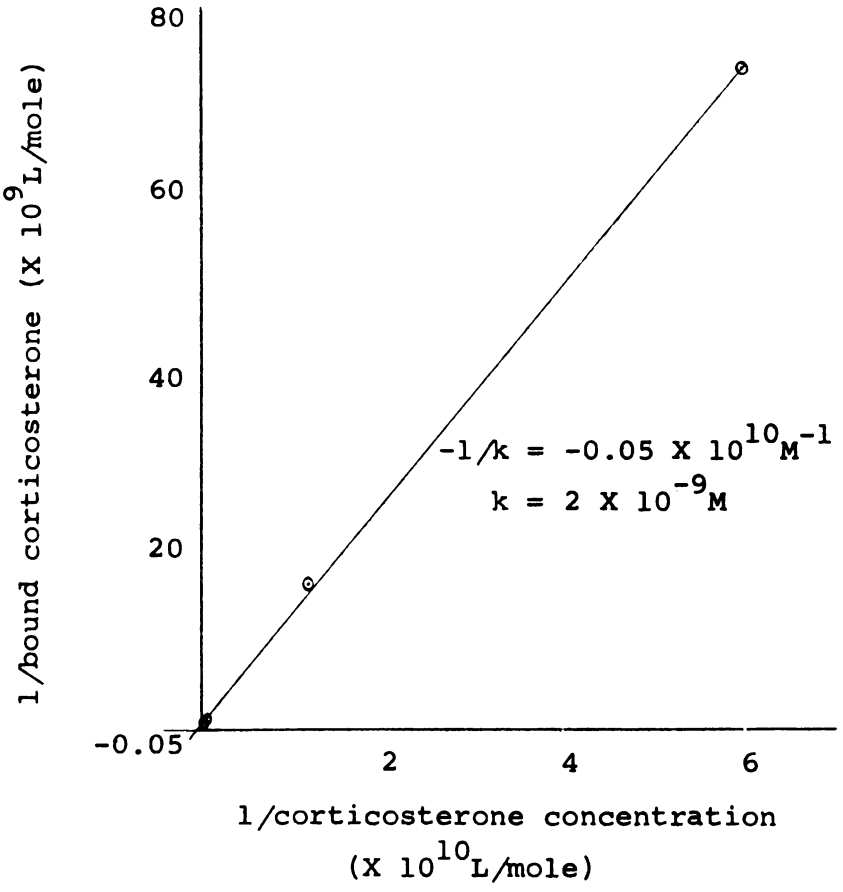
80
60
40
20
-0.05
2
4
6
1/bound corticosterone (X 10^{9} L/mole)
1/corticosterone concentration
(X 10^{10} L/mole)
$-1/k = -0.05 \times 10^{10} M^{-1}$
$k = 2 \times 10^{-9} M$

Figure 10

Fig. 11.--Scatchard plot of data represented in Figure 10.

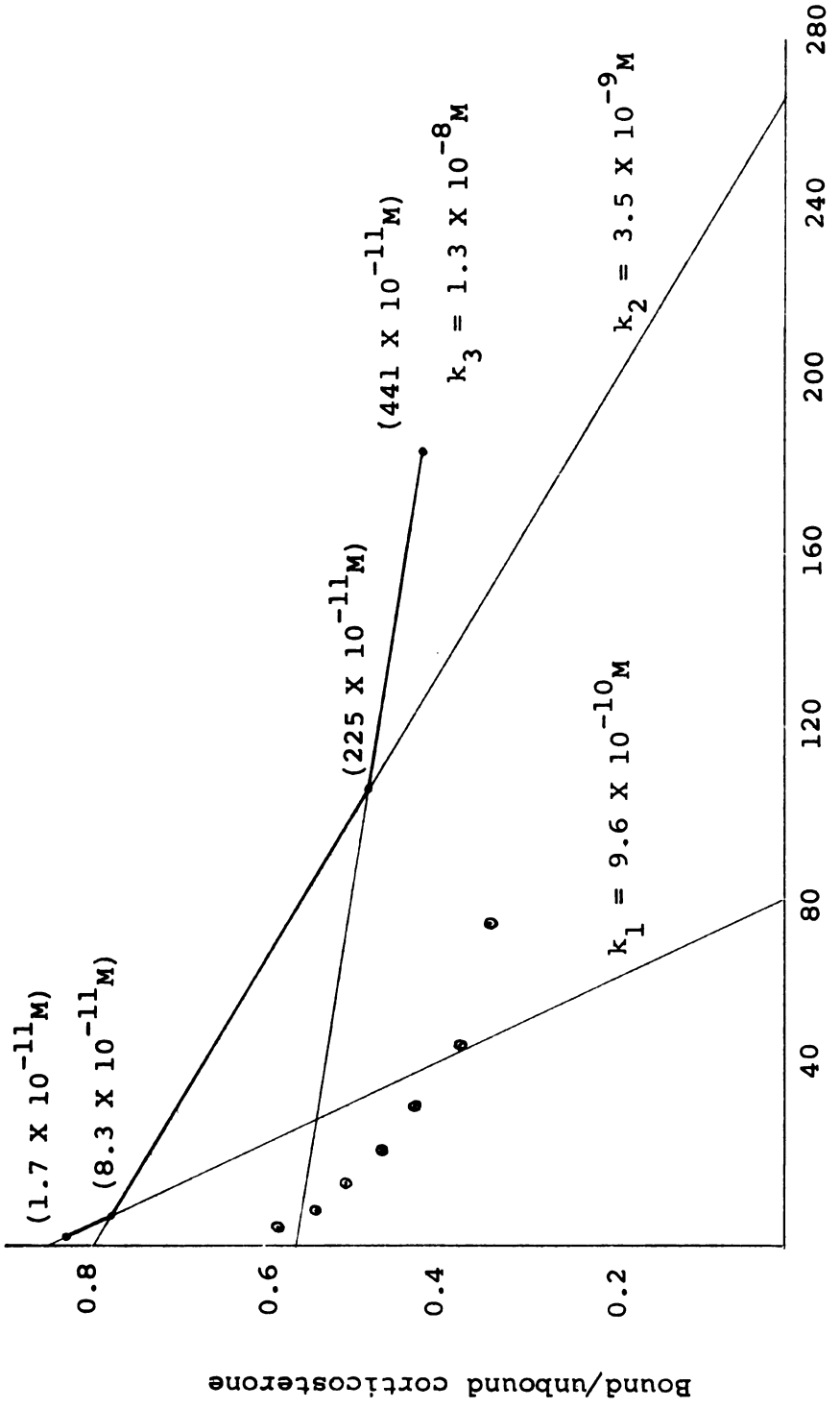
Bound/unbound corticosterone
0.8
0.6
0.4
0.2
40
80
120
160
200
240
280
X 10^{-11}M corticosterone bound
(1.7 X 10^{-11}M)
(8.3 X 10^{-11}M)
(225 X 10^{-11}M)
(441 X 10^{-11}M)
k_1 = 9.6 X 10^{-10}M
k_2 = 3.5 X 10^{-9}M
k_3 = 1.3 X 10^{-8}M

Figure 11

represent either a transition between high and low affinity binding or a family of steroid binding proteins or binding sites having progressively lower affinity for corticosterone. Different affinity binding sites were proposed by Tucker et al. (98) for mammary cells. They had more points from which to derive lines and concluded there were two sites of relatively high affinity for hydrocortisone in mammary cells in culture. Their highest affinity site had a k of 2×10^{-9}M which is close to the intermediate binding constant in this experiment. The presence of more than two binding constants due to either other proteins or changes in binding affinity with progressive increase in steroid bound cannot be ruled out.

The second treatment of the Figure 11 Scatchard plot involved drawing a curve through the first three points and subtracting the low affinity slope. This does little for the data as shown by the circled points in the figure since a new curve was formed rather than a straight line.

The binding constants derived from the two sets of data ranged between 0.96×10^{-9}M and 5.9×10^{-9}M. The double reciprocal and Scatchard plots both gave similar binding constants with the high affinity portions of the

curves ranging between 0.96 X 10^{-9}M and 2.1 X 10^{-9}M with the Scatchard plots and 1 and 4 X 10^{-9}M with the double reciprocal plots.

As an attempt to further define the high affinity binding constant, or constants, an experiment was performed using twelve corticosterone concentrations and two different bovine mammary tissues with one of them duplicated. The twelve corticosterone-H^3 concentrations are shown in Table 18. After equilibrium dialysis at these concentrations aliquots were counted for tritium and from that data bound to unbound ratios and concentration of bound corticosterone were calculated and subjected to statistical analysis.

TABLE 18.--Twelve corticosterone concentrations used in equilibrium dialysis of 60-80% saturated ammonium sulfate fractions from two bovine mammary tissues.

Tube	X 10^{-11}M
1	1.66
2	3.32
3	4.98
4	9.96
5	20
6	53
7	138.9
8	226.4
9	312
10	442.9
11	2174
12	4339

For statistical purposes each dialysis sac represented one value for bound to unbound ratio and one value for concentration of steroid bound. That made six points available for each axis of the Scatchard plot for each steroid concentration used. An analysis of variance was performed on each group of six points to determine whether variation between experiments was significantly larger than variation within an experiment. A significant difference between experiments was seen in only one case where the second lowest steroid concentration in one experiment had points which were obviously different from any of the others. These data were discarded before further calculations were made. After that deletion there were no significant differences between experiments so the points at each concentration were averaged before plotting by Scatchard's method (Figure 12). Figure 12 shows sample standard deviations for both bound to unbound ratio and concentration of steroid bound for only the first five concentration points since after the fifth concentration point the curve was almost horizontal. As can be seen in that figure the standard deviations were rather large. The large variation was traceable to flocculation of some material in the labeled

Fig. 12.--Scatchard plot of combined 60-80 ammonium sulfate fraction, equilibrium dialysis corticosterone-binding data from two tissues with duplication of one. Two ml diluted (1:9 TMK) 60-80% saturated ammonium sulfate fraction per dialysis sac, two sacs per 100 ml total volume containing 1.66×10^{-11}M corticosterone-H^3 and different levels of unlabeled corticosterone. Dialyzed 24 hours at 4°C. Means and standard deviations for bound/unbound and concentration bound are shown.

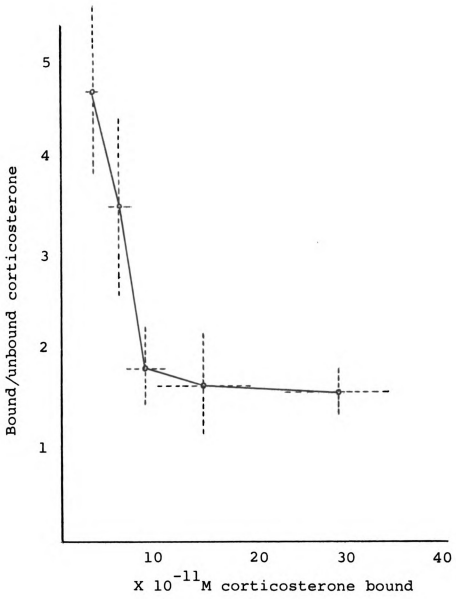
Bound/unbound corticosterone
5
4
3
2
1
10
20
30
40
X 10^{-11}M corticosterone bound

Figure 12

corticosterone used in these experiments. The flocculation produced error in sampling for counting but although the standard deviations were relatively large they were such that they had little effect upon the Scatchard plot slope. That is, in the high affinity portion of the Scatchard curve most of the variation was in the bound to unbound term and as such there was little effect on the slope. Variation in the low affinity portion of the curve was mostly in the amount bound which produced little effect on the slope representative of that low affinity binding.

The binding constants and their standard deviations, calculated from the data represented in Figure 12, are shown in Table 19. The slope of each segment of the Scatchard curve was then tested (109) to determine whether it was significantly different from the slope of its succeeding segment. From left to right in Figure 12 the first slope was significantly different from the second, primarily because the means of the duplicates were almost equal, and the second from the third. The slope of the third segment was significantly different from that of the fourth segment ($P=0.10$) but thereafter none of the

TABLE 19.--Binding constants, k, and their standard deviations, S_k, calculated for the five segments of the curve shown in Figure 12.

k		S_k
2.332×10^{-11}M	±	0.256×10^{-11}M
1.516×10^{-11}M		0.032×10^{-11}M
35.238×10^{-11}M		12.189×10^{-11}M
220.504×10^{-11}M		183.676×10^{-11}M
401.140×10^{-11}M		402.746×10^{-11}M

slopes was significantly different from the slope of its adjacent segment.

Because of the large standard deviations involved the slope of the first segment of the curve was not used alone to determine the highest affinity binding constant. As an alternative the data for the first three concentration points of each experiment were used to determine a binding constant by calculation of a linear regression equation for each experiment. By doing that the differences between experiments could again be tested for statistical significance. The binding constants, which are the reciprocals of the regression coefficients, and their

standard deviations are shown in Table 20. When the slopes from which the binding constants were derived were tested for significant differences none of the slopes was significantly different from any of the others. The data could therefore be combined to give an average binding constant of 3.2×10^{-11}M.

TABLE 20.--High affinity binding constants and their standard deviations determined by linear regression calculations on data represented in Figure 12.

Tissue	k	±	S_k
	----------X 10^{-11}M----------		
1	2.64		1.34
2	3.57		1.35
3	3.40		1.79

The data obtained in these last three experiments did not resolve the question of whether there are several high affinity binding sites of differing affinities. However, there was a striking aspect to the data in that the binding constants calculated were much lower than previously observed. They were of the order of 10^{-11}M

rather than the 10^{-9}M determined previously. One possible explanation is the greater number of low-steroid concentration points used in the latter experiments. These apparently enabled better definition of the high affinity binding constant. There is, however, no reason to believe that lower binding constants could not be determined with even lower steroid concentrations. However, any attempt to use lower steroid concentration would soon be limited by the specific activity of the tritiated corticosterone available. The 30 Ci/mM corticosterone used in these experiments could not be used very much below the 1.66×10^{-11}M concentration used as the lowest steroid concentration. It therefore became limiting in the determination of the lowest possible binding constant.

GENERAL DISCUSSION

A number of tissues have been shown to bind glucocorticoids, including mammary cells in culture, and glucocorticoids are also known to be essential to the process of lactation. Since binding of a hormone in a cell implies a biological function of that hormone in that cell, and since glucocorticoids are so important to lactation, there is a strong possibility that mammary tissue contains a material able to specifically bind adrenal glucocorticoids. This study has shown the presence of such binding protein or proteins in mammary tissue.

Mammary glucocorticoid binding was observed in centrifugally separated nuclear, mitochondrial and microsomal particulate fractions although the real extent of binding in each of the particulate fractions could not be accurately determined in these experiments. The desired accuracy was not achieved because the methodology used to study binding in the particulate fractions allowed

dissociation and some cross-contamination among fractions. Therefore the data concerning distribution among fractions should be considered only approximate.

Two conclusions can be drawn from the work on particulate distribution of binding. One is that such binding did exist and the second is that the binding material was extractable by high pH. The observation that high pH is required to extract the binding protein may indicate that the protein is an integral part of the particulate structure and extractable only by rigorous treatment such as sodium hydroxide. The particulate binding observed in these studies may be similar to rat liver mitochondrial glucocorticoid binding reported by DeVenuto and Muldoon (81). When their mitochondrial preparations were sonicated and centrifuged 105,000 x g over 91% of the binding material sedimented thereby indicating that such binding protein was an integral part of the mitochondrial structure.

Observations on the stability of the binding protein to freezing were made on two of the fractions, the sodium hydroxide extract and the 60-80% saturated ammonium sulfate fraction of the 30,000 x g supernatant. Neither lost their ability to bind steroids. The ammonium sulfate

fractions were used for competition and binding constant studies and when equilibrium dialyzed at equal steroid concentrations (low concentration point in Figure 5) the bound to unbound ratios were all in reasonable agreement thereby indicating the freezing and thawing had little effect on the high affinity binding activity in the 60-80% saturated ammonium sulfate fraction.

Several fractionation methods were attempted during this study and the most important fractionation step was ammonium sulfate precipitation. It was used both before and after incubation with tritiated steroid but although ammonium sulfate precipitation of steroid-labeled protein has been used (108) to study degree of binding activity, supposedly with little loss of label, this study has shown that very low concentrations of glucocorticoid will precipitate with denatured protein during ammonium sulfate treatment. Therefore, ammonium sulfate fractionation should be used before incubation with steroid if accurate measure of binding is to be made. Used in such a manner, it was a very successful method for partial purification of the cytoplasmic high affinity binding protein which became the fraction of primary interest in this study. The soluble high affinity steroid binding

activity was concentrated in the 70-80% ammonium sulfate fraction but was also high in the 60-70% fraction. Therefore, the 60-80% fraction was used for competition and binding kinetcis studies.

After ammonium sulfate precipitation, further fractionation was attempted. Calcium phosphate gel and Sephadex G-100 filtration added little to the purification of the 60-80% saturated ammonium sulfate fraction. DEAE-cellulose ion exchange chromatography did provide good separation of a number of protein fractions, one of which had much higher specific binding activity than the others. Even finer separation might have been obtained on the DEAE had a more shallow gradient been used during elution.

Studies of binding kinetics and competition among steroids were studied in the 60-80% saturated ammonium sulfate fraction of the 30,000 x g supernatant. Previous studies, in the particulate fractions, had indicated steroid binding to be linear with steroid concentration. The reason for that linear uptake was probably due to the presence of two or more types of binding sites, each with different affinities for corticosterone. That situation was clarified when the binding data for the 30,000 x g

supernatant was plotted by Scatchard's method. The Scatchard plot showed low affinity binding sites which appeared to be unsaturable and have linear steroid uptake. Such sites were apparently responsible for the "infinite" binding observed in the particulate fractions (Figure 5 and Table 13). The existence of such a phenomenon has been observed in other tissues. Gardner and Tomkins (87), during isolation of a corticosteroid-binding macromolecule from hepatoma cells, observed an almost linear increase in binding until added steroid concentration exceeded 10^{-5}M. Nonspecific, low affinity, binding which increased linearly with increasing steroid concentration was also seen in hepatoma cells (57) with similar observations having been made with aldosterone in rat kidney (110) and in thymus cells using a number of glucocorticoids and other steroids (92). The "infinite" binding observed in this study was probably of the same low affinity, nonspecific nature as seen in those other tissues. This was further confirmed when Tucker et al. (98) showed low affinity binding in their mammary cell cultures.

The competition experiments revealed that hydrocortisone was bound equally as well as corticosterone although some rat liver work (81,82) showed corticosterone

preferentially bound compared to hydrocortisone. One explanation could be tissue differences but species difference is also a feasible explanation since corticosterone is the glucocorticoid found in the rat while both corticosterone and hydrocortisone are present in the cow. If tissue difference is unimportant, the rat corticosteroid-binding protein must be different from that of the bovine and purification of rat mammary binding fractions and testing them for corticosterone-hydrocortisone competition should answer whether tissue or species differences predominate.

One other factor should be considered when pondering rat vs bovine steroid binding. The liver is the main site of metabolic degradation of glucocorticoids and as such it may have different receptors, for different purposes, than the mammary gland. The rat liver receptors may be designed solely for the degradation of corticosterone. The rat would have no need to degrade hydrocortisone and it may therefore not have receptors for hydrocortisone.

Estrogen uptake by the mammary gland has been shown previously (111) and estradiol-C^{14} was bound in the 750 x g supernatant in this study but competition

experiments on the 60-80% saturated ammonium sulfate fractions showed estradiol noncompetitive for corticosterone binding sites (Figure 6). These experiments do not indicate whether or not estradiol is binding to some portion of that 60-80% saturated ammonium sulfate fraction but only that it doesn't compete with corticosterone.

Progesterone competed with corticosterone for binding sites and the competition was extensive though not complete. Progesterone will bind to hydrocortisone receptors in hepatoma cells (57,87) and bovine mammary cells in culture (97,98) but the physiological significance is unknown. A physiological relationship is suggested by the fact that progesterone rises to a high level during pregnancy but markedly decreases after paturition while at parturition free glucocorticoid levels increase. Perhaps during pregnancy the high progesterone levels promote extensive binding of progesterone to the glucocorticoid receptors and progesterone on the receptors might make the binding protein unable to express any lactational influences it is assumed to have. At parturition the combined decrease of progesterone and increase in glucocorticoid would promote glucocorticoid binding. The

bound glucocorticoid would then enable the receptor protein to exert its lactogenic effect.

The binding constants for the soluble cell fraction (using the 60-80 ammonium sulfate fraction) under equilibrium conditions were first determined to be 0.96×10^{-9} to 5.9×10^{-9}M for the high affinity portion of the binding curve while the low affinity portion of the Scatchard plots was almost horizontal and a low affinity binding constant was impossible to calculate. The transition area, between the high and low affinity portions of the curve, was difficult to deal with. It could represent either a family of binding proteins or binding sites, or merely transition between high and low affinity binding. Tucker et al. concluded there were at least two sites with relatively high affinity binding in their mammary cells in culture (98). With more than one site available any binding and competition data collected at steroid concentrations in the transition area gives misleading information on the real nature of the binding. The $k = 5.9 \times 10^{-9}$M given above probably represented influence of low affinity binding by considering too much of the low affinity portion in the transition portion of the Scatchard plot.

Estimates of the binding constants from the charcoal dialysis experiment shown in Figure 7 gave k's of 4.4×10^{-9}M to 2.5×10^{-8}M. The 4.4×10^{-9}M value is within the range found by equilibrium dialysis.

Those binding constants can be compared to corticosterone binding globulin (CBG) which has $k = 3 \times 10^{-8}$M at 37°C and 2×10^{-9}M at 4°C (6). Those first binding constants for the mammary binding protein were obtained at 4°C and are very close to the CBG value. A lower k value in the mammary gland would encourage transfer of glucocorticoid from the blood CBG to mammary gland and the CBG could be considered a vehicle for transport of glucocorticoid to the mammary gland; however, the similar binding affinities would not indicate such a role for CBG. With similar affinity for the same steroid they would be in competition for that steroid therefore the decrease in CBG levels observed soon after parturition may be the means by which glucocorticoid is made available to the mammary gland for lactogenesis.

Similar binding constants could indicate another, less important, role for CBG in relation to the mammary binding. Blood glucocorticoid levels can be increased by stress and doubled during normal diurnal cycles. Much

of those sharp increases in corticoid is probably absorbed by CBG because with similar binding constants distribution of the increase between CBG and the mammary binding protein would be proportional to the quantity of each. Distribution between the two would save the mammary receptors from really large variations in glucocorticoid concentration and if the glucocorticoid receptors are important in enzyme induction such a mechanism protects the cell from rapid or cyclic changes in enzyme levels when such changes would not necessarily be beneficial to the cell or tissue.

Such relationships between CBG and the mammary binding protein are very feasible with a k of approximately 10^{-9}M. However, the last data collected indicated a much lower value for k. A lower k value necessitates revision of the hypotheses proposed to relate CBG and the binding protein. It was mentioned that a k value lower for mammary binding protein than CBG would encourage transfer of corticosteroid from CBG to the mammary binding protein. Because of the approximately 100-fold higher affinity the mammary protein would be less sensitive to normal physiological changes in blood glucocorticoid concentration and expression of its physiological effect may

therefore be more dependent on glucocorticoid-progesterone ratio than absolute concentration.

One important factor should be considered when discussing the lower k value. It should be remembered that the lower binding constant was obtained with very low glucocorticoid concentrations and dialysis of longer duration. If the glucocorticoid exerts any sort of protective effect upon the binding protein those two factors, low steroid concentration and longer time could act in conjunction to produce an artificially low k value.

If the lower k is correct, however, the cytoplasmic binding protein has a very high affinity for glucocorticoid and one can speculate whether it plays a passive or and active role in the initiation of lactation. In a passive role it might act merely as an "intracellular CBG" whose function would be transport of glucocorticoid through the cytoplasm to the nucleus similar to the cytoplasmic estrogen-binder observed in uterine tissue. A nuclear role for glucocorticoid seems to be implied by the requirement for glucocorticoid during cell division preceding casein synthesis. Such a nuclear role implies existence of a nuclear receptor for the glucocorticoid and if the nuclear receptor is not merely the cytoplasmic

binding protein in a new role it probably has a binding constant even lower than 3 X 10^{-11}M in order to facilitate transfer of glucocorticoid from cytoplasmic binder to nuclear receptor. Any material with a k even lower than 3 X 10^{-11}M would be very difficult to study accurately with presently available isotopes.

The cytoplasmic binding protein could also have a direct extranuclear role in lactation. The observation that certain glucocorticoids promote rough endoplasmic reticulum formation makes such speculation feasible. The binding protein could conceivably play some role in the interaction of polysomes and smooth endoplasmic reticulum. Further speculation envisions the binding protein as some vital enzyme which is inactive when associated with progesterone but active when bound to glucocorticoid. Or perhaps the glucocorticoid promotes the association between two proteins required for enzyme activity. An example would be the association of UDP-galactosyl transferase and α-lactalbumin to create lactose synthetase. Lactose synthetase activity appears to be sensitive to progesterone-glucocorticoid levels during pregnancy and parturition and although any glucocorticoid effect on those enzyme levels could be nuclear that enzyme would be

the prime candidate for further study of the role of the cytoplasmic glucocorticoid-progesterone binding protein.

Relationships existing among CBG, glucocorticoid, progesterone, mammary gland binding protein and lactation have been partially clarified by this study. The existence of a binding protein(s) in particulate and cytoplasmic fractions has been shown in mammary gland. It will bind corticosterone, hydrocortisone, and progesterone and has a binding constant best estimated as 3.2×10^{-11} M for corticosterone.

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APPENDIX

APPENDIX TABLE 1.--Chemical Formulation for Disc Electrophoresis (Canalco Formulation)*

Stock Solutions

Component		Quantity	Component		Quantity
A)	1 N HCl	48 ml	E)	Riboflavin	4 mg
	Tris**	36.3 g		H_2O	100 ml
	Temed***	0.23 ml			
	H_2O	to 100 ml	F)	Sucrose	40 g
				H_2O	to 100 ml
B)	1 N HCl	48 ml			
	Tris	5.98 g	G)	Ammonium	
	Temed	0.46 ml		persulfate	0.14 g
	H_2O	to 100 ml		H_2O	100 ml
C)	Acrylamide	28 g	H)	10X buffer	
	Bis****	0.735 g		Tris	3.0 g
	H_2O	to 100 ml		Glycine	14.4 g
				H_2O	to 1000 ml
D)	Acrylamide	10 g			
	Bis	2.5 g			
	H_2O	to 100 ml			

Working Solutions

Separating Gel	Stacking Gel
1 part A	1 part B
2 parts C	2 parts D
1 part H_2O	1 part E
(mix 1:1 with G to polymerize)	4 parts F
	(expose to fluorescent light to polymerize)

*Standard Gel (7%)--stacks at pH 8.9, runs at pH 9.5.
**Tris = 2-amino-2-hydroxymethyl-1,3-propanediol.
***Temed = N,N,N',N'-tetramethylethylenediamine.
****Bis = N,N'-methylenebisacrylamide.

APPENDIX TABLE 2.--Composition of scintillation fluid.

Component	Quantity
Dioxane	770 ml
Xylene	770 ml
Absolute ethanol	460 ml
Naphthalene	160 g
PPO*	10 g
POPOP**	0.1 g

*2,5-diphenyloxazole.
**1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene.