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Cytosol Binding of Steroid Hormones
in Sheep Brains and Pituitaries

A manuscript
of a Journal Article
Presented to the
Department of Zoology
Brigham Young University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by
David Carl Marcusen

April 1977

This manuscript by David Carl Marcusen is accepted in its present form by the Department of Zoology of Brigham Young University as satisfying the thesis requirement for the degree of Master of Science.

19 Mar 77
Date

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Cytosol Binding of Steroid Hormones
in Sheep Brains and Pituitaries¹

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Running Title: Steroid Binding in Sheep Brains.

ABSTRACT

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The uptake and binding of tritiated cortisol, corticosterone, and dexamethasone in cytosols isolated from various regions of non-adrenalectomized (intact) and adrenalectomized sheep brains and pituitaries were studied. [^3H]-cortisol and [^3H]-corticosterone were bound preferentially by cytosols from the hippocampal and septal regions of the brain, whereas, [^3H]-dexamethasone was preferentially bound by cytosol from the pituitary. Cytosols isolated from the brains of adrenalectomized sheep bound significantly more [^3H]-corticosterone than did cytosol from the brains of intact animals. Pre-incubation of cytosols from the hippocampus of adrenalectomized animals with non-radioactive testosterone or estradiol did not significantly block the subsequent binding of [^3H]-cortisol. Results of pre-incubations with non-radioactive progesterone were non-reproducible with a large standard deviation among replicates. These findings provide evidence that the sites of feedback inhibition are the same in the sheep as described for other mammalian systems, and that in the sheep as in other mammals the principle site of localization of cortisol and corticosterone is in limbic structures of the brain, whereas, the principle site of uptake of dexamethasone is in the pituitary.

INTRODUCTION

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Divergent opinions exist regarding the exact locations where glucocorticoids bind to facilitate negative feedback phenomena. Early studies indicate that the glucocorticoids are bound by and have a direct effect on the anterior pituitary (De Weid, 1961; Russel *et al.*, 1969; Roberts and Keller, 1955; Rochefort *et al.*, Royce and Sayers, 1959). Others have shown that they are bound by the hypothalamus (Chowers *et al.*, 1965; Davidson and Feldman, 1963; Davidson *et al.* 1965; Davidson *et al.*, 1968; Endroczi *et al.*, 1961; Kendall *et al.*, 1964). Later experiments using scintillation and histofluorescence as well as enzyme studies and autoradiographic techniques (Rhees, *et al.*, 1972; Stevens, *et al.* 1971; Davidson and Feldman, 1967; Kawakawi *et al.*, 1968; McHugh and Smith 1967; Stumpf, 1971a) all indicate that limbic structures, particularly the hippocampus and septum, are the primary target areas concerned with the binding of glucocorticoids in rats.

It is possible that species differences exist concerning the control of the levels of glucocorticoids in the plasma. For example, early studies have shown that the median eminence and adjacent regions in the rat and dog are effective loci for lesions which block ACTH secretion (Ganong *et al.*, 1961; De Wied, 1961), whereas the mammillary bodies and the posterior portion of the tuberal region

apparently play a similar role in rabbits (deGroot and Harris, 1950). In more recent studies most researchers agree that in rats, monkeys, cats, and rabbits the limbic regions, particularly the hippocampus and septum, are where glucocorticoids control the production and release of CRF and ACTH (Rhees *et al.* 1975a; Grosser *et al.*, 1973; McEwen and Wallach, 1973; DeKloet *et al.*, 1975; Krieger, 1973; Knizley, 1972; Gerlach *et al.* 1976; Stevens *et al.*, 1973). DeKloet *et al.* (1975), Grosser and Stevens (1976) and Rhees *et al.* (1975b) recently found that cytosol isolated from the anterior pituitary bound labeled dexamethasone preferentially. In the same study they also reaffirmed that corticosterone was bound in significantly greater quantities in the hippocampus and septum than it was in the hypothalamus and anterior pituitary.

Sheep were chosen as the experimental animals for the studies described here. Sheep are relatively large mammals whose predominant glucocorticoid is cortisol which is also the major glucocorticoid in man. The interest was to determine whether or not the data obtained in other mammals (particularly the rat whose predominant glucocorticoid is corticosterone) could be extended to include the sheep and cortisol, and to determine if labeled dexamethasone and corticosterone were preferentially bound by certain areas of the sheep brain or pituitary.

The data presented in this report are results of

in vitro experiments designed to determine whether or not there is preferential binding of labeled cortisol, corticosterone, or dexamethasone by cytosol isolated from various areas of the sheep brain and pituitary, and to determine the range of specificity of the receptor sites.

MATERIALS AND METHODS

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One hour prior to surgery adult western crossbred ewes were tranquilized with ace-promazine (20 mg/animal) and under a local anesthetic (1% lidocaine) were adrenalectomized. Two dorsal incisions just posterior to the rib cage were made and the adrenal glands were dissected out and removed. They were maintained on physiological saline and alfalfa pellets for three to five days after surgery. Then, under sodium pentobarbital anesthesia the animals were perfused with 5% sucrose in a 0.9% saline solution via the carotid vessels and exsanguinated via the jugular veins. Brains and pituitaries were quickly removed, placed on ice and the brain dissected into septum, hippocampus, hypothalamus, cortex, brain stem, cerebellum, and a representative sample from all remaining tissue (remainder). Each tissue was homogenized in a teflon-glass homogenizer in eight volumes of ice-cold Tris-EDTA (Tris 0.01 M- EDTA 0.0015 M, pH 7.4) buffer and the resulting homogenate centrifuged at 105,000 x g at 4° C for one hour. One ml aliquots of the 105,000 x g supernatant fraction (cytosol) were incubated for four hours at 4° C in the presence of 5×10^{-9} M of either [1, 2, 6, 7-³H]-cortisol (91 ci/m mole), [1, 2, 6, 7-³H]-corticosterone (87 ci/m mole), or [1, 2, 4-³H]-dexamethasone (22.6 ci/m mole). The radioactive steroids were obtained from New England Nuclear, Boston, Massachusetts.

To determine the effects of endogenous cortisol on the saturation of receptor sites, brain cytosols obtained from non-adrenalectomized (intact) sheep were incubated with labeled steroid hormones as described above. In addition, aliquots of cytosol from adrenalectomized animals were incubated (pre-incubated) for one-half hour at 4° C with 5 X 10⁻⁶M non-radioactive cortisol, corticosterone, cortisone, progesterone, estradiol, or testosterone prior to being incubated with labeled cortisol as described above to study further the specificity of the [³H]-cortisol binding receptors. The unlabeled steroids were obtained from the Sigma Chemical Company.

Following incubation the free labeled steroid was separated from the bound labeled steroid using a modified Baxter and Tomkins' (1971) activated charcoal assay. Activated charcoal was washed in 6N HCl, rinsed in distilled water, neutralized to pH 7.0, filtered, and dried. The charcoal (100 mg/ml) was added to 1% dextran Tris-EDTA buffers and kept under constant stirring at 4° C. A 400 µl aliquot of the cytosol was placed in a disposable glass culture tube, 50 µl of the charcoal buffer solution were added to each tube, and the contents vortexed for ten seconds and centrifuged at 600 x g for five minutes, after which the supernatant was removed and recentrifuged at 1000 x g for five minutes. Two 100 µl samples of the supernatant were taken for scintillation counting and protein assays. One

sample was added to aquasol and assayed for radioactivity in an Isocap/300 liquid scintillation counter. The other sample was assayed for protein content by the technique of Lowry *et al.*, (1951) using bovine serum albumin as standard. Results were expressed in fmoles of radioactive steroid bound per mg of protein.

Significant of difference in the amount of labeled steroid binding between the same brain areas of intact and adrenalectomized animals was determined by a two-tailed t-test. Significance of difference in binding between different brain areas was determined by a Neuman-Keuls sequential range test.

RESULTS

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Bound [³H]-cortisol in brain cytosols from adrenalectomized sheep can be grouped into the following three categories: (1) the septum and hippocampus demonstrated preferential binding over all other areas; (2) a moderate amount of binding occurred in the hypothalamus and cortex; and (3) there was relatively little binding in the brain stem, cerebellum, and remainder (Fig 1). There was significantly less cortisol bound in cytosol from intact sheep when compared with that which was bound in the brain cytosols from adrenalectomized animals (Fig 1). This difference was greatest in the septum and hippocampus. There was a decrease in the amount of labeled hormone bound in the brain cytosols from animals that had been perfused when compared with the non-perfused animals (Fig 2). However, this difference was not statistically significant. ($p < .05$).

In cytosols isolated from the hippocampus of adrenalectomized sheep, pre-incubation with unlabeled cortisol, corticosterone, and cortisone almost completely blocked the subsequent uptake of [³H]-cortisol. Even less [³H]-cortisol was bound in the cytosol pre-incubated with unlabeled cortisol, corticosterone, and cortisone than there was in the cytosols from intact sheep whose endogenous cortisol was still present (Fig 3). Pre-incubation with progesterone had varied effects on subsequent [³H]-cortisol

binding with varying results. Testosterone and estradiol pre-incubations had little effect on labeled cortisol binding (Fig 3).

Cytosols isolated from the hippocampal and septal areas of sheep brains bound [³H]-cortisol and [³H]-corticosterone in significantly greater amounts when compared to the amount of [³H]-dexamethasone bound in the same areas (Fig 4). Cytosol from the cortex, brain stem, cerebellum, and remainder bound [³H]-cortisol, [³H]-corticosterone, and [³H]-dexamethasone in approximately equal amounts. Cytosols isolated from the hypothalamus and pituitary were found to bind significantly ($p < .05$) greater amounts of [³H]-dexamethasone than either [³H]-cortisol or [³H]-corticosterone (Fig 4).

The amount of [³H]-dexamethasone bound per mg protein in the cytosol from the pituitary was not significantly different from [³H]-cortisol binding in the septum and hippocampus, however, pituitary cytosol was found to bind only small amounts of [³H]-cortisol and [³H]-corticosterone (Fig 4).

DISCUSSION

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These data indicate a finite number of receptor sites in brain and pituitary cytosols capable of binding glucocorticoids. In adrenalectomized animals the number of sites are greatly increased due to the lack of endogenous cortisol. Endogenous cortisol does not completely saturate the binding sites because hippocampal cytosol from the intact sheep bound more [³H]-cortisol than did hippocampal cytosol from the adrenalectomized sheep which had been pre-incubated with a 1000 fold excess of the unlabeled hormone. The population of binding sites for cortisol being concentrated in the hippocampus and septum are different than those for dexamethasone which are concentrated in the anterior pituitary. The presence of different receptors in various brain areas could be due to the presence of different cell types in these areas.

With respect to the hippocampal and septal binding of cortisol, corticosterone, and cortisone, results obtained in this study compliment and extend observations reported in the literature. Biochemical and autoradiographic data (Grosser *et al.*, 1971; McEwen, 1970; Stevens *et al.*, 1971; Grosser *et al.*, 1973; Gerlach *et al.*, 1972; Stumpf, 1971a; Stumpf, 1971b; Stumpf and Sar, 1972; Stumpf and Sar, 1973; and Rhee *et al.*, 1975) demonstrate that labeled corticosterone is concentrated in and bound by

specific cells of rat brains. Using autoradiographic techniques Gerlach *et al.*, (1976) found that in monkeys infused with [³H]-corticosterone cells were labeled both in the pyramidal neurons of the cornu ammonis and in the granule cells of the gyrus dentatus. [³H]-dexamethasone was bound in cytosols from rat pituitaries in larger amounts than either [³H]-cortisol or [³H]-corticosterone (DeKloet *et al.*, 1975). There was a population of receptor sites in the hypothalamus of sheep as well as the pituitary. Interestingly, corticosterone injections in rats (Dallman and Jones, 1973) and cortisol, corticosterone, and dexamethasone injections in rhesus monkeys (DeKloet *et al.*, 1975; and McHugh and Smith, 1967) all inhibit stress induced ACTH release. However, when intrahippocampal implants of cortisol, corticosterone and dexamethasone were made in the rabbit only the cortisol and corticosterone implants inhibited ACTH release from the anterior pituitary (McEwen *et al.*, 1972). These data from the literature and those obtained from the sheep indicate that dexamethasone must be bound in sites other than the hippocampus (i.e. the anterior pituitary and hypothalamus) to exert the negative feedback phenomena of the pituitary-adrenal axis demonstrated by injections of dexamethasone. The limbic structures of the brain (i.e. hippocampus and septum) are the primary sites for the binding of the

naturally occurring hormones cortisol and corticosterone.

Extension of these data to include a variety of larger mammals is important to the knowledge of somatic and behavioral functions in the monkeys and humans. Many studies have shown that glucocorticoids effect somatic and behavioral functions by direct action on the central nervous system (McEwen *et al.*, 1975). It is not completely understood what the changes in electrical activity exhibited by neurons which retain the glucocorticoids might have on the negative feedback system (Pfaff *et al.*, 1974). It is known that glucocorticoids have been shown to have behavioral effects such as alterations of sensory input, suppression of conditioned avoidance behavior and the occurrence of paradoxical sleep (McEwen *et al.*, 1975).

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FIGURES

- Figure 1. Comparison between [^3H]-cortisol binding in cytosol isolated from various areas of brains from adrenalectomized and intact animals (column = mean, vertical line = \pm S.E.).
- Figure 2. Comparison of [^3H]-cortisol binding in various brain areas of perfused and non-perfused brains of adrenalectomized sheep (column = mean, vertical line = \pm S.E.).
- Figure 3. Effects of pre-incubation with various unlabeled steroid hormones on the subsequent binding of [^3H]-cortisol in cytosols isolated from the hippocampus of brains from adrenalectomized sheep (column = mean, vertical line = \pm S.E.).
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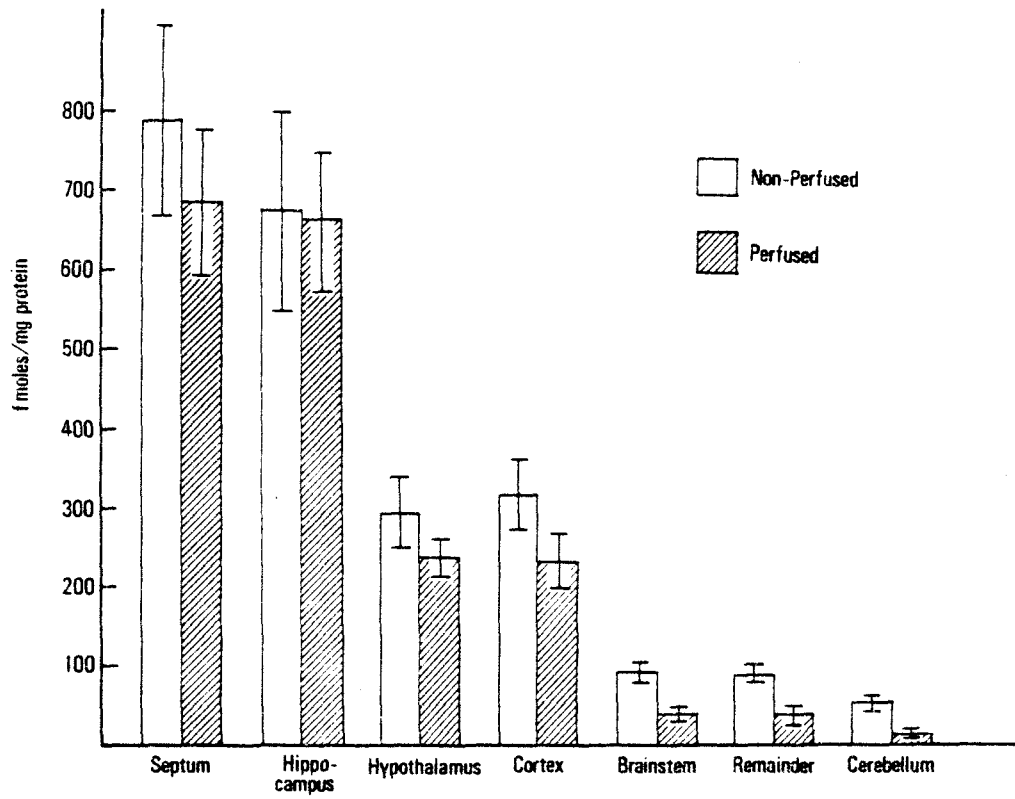


Figure 1
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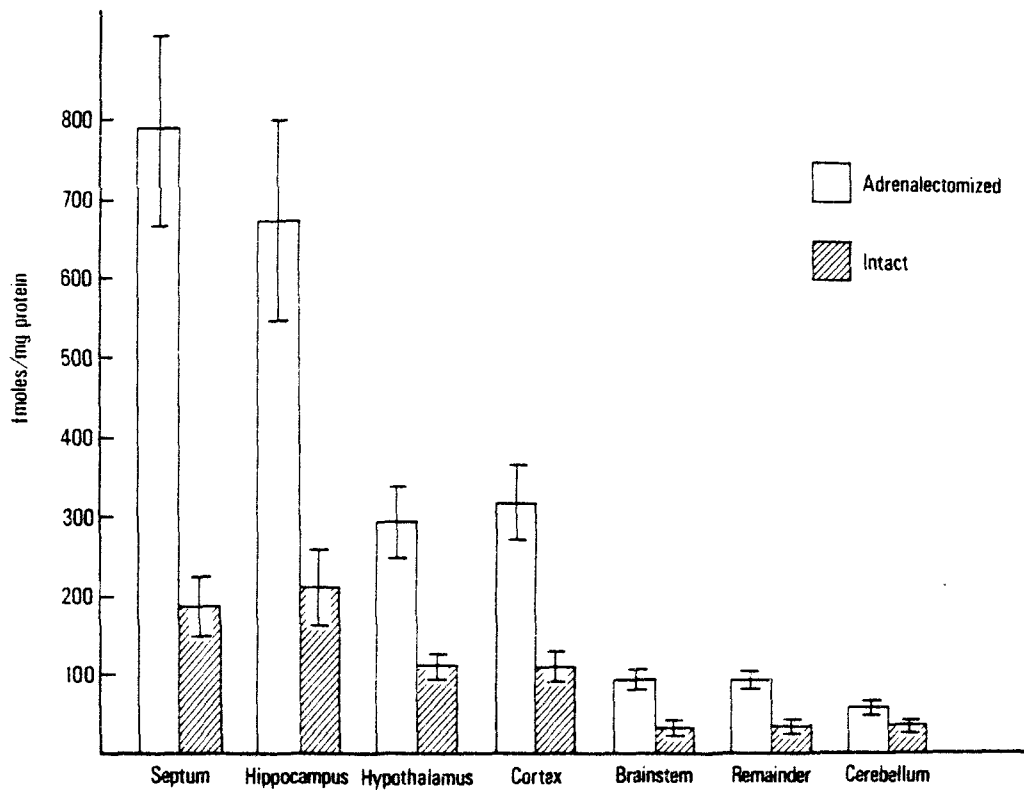


Figure 2
Marcusen and Rhees

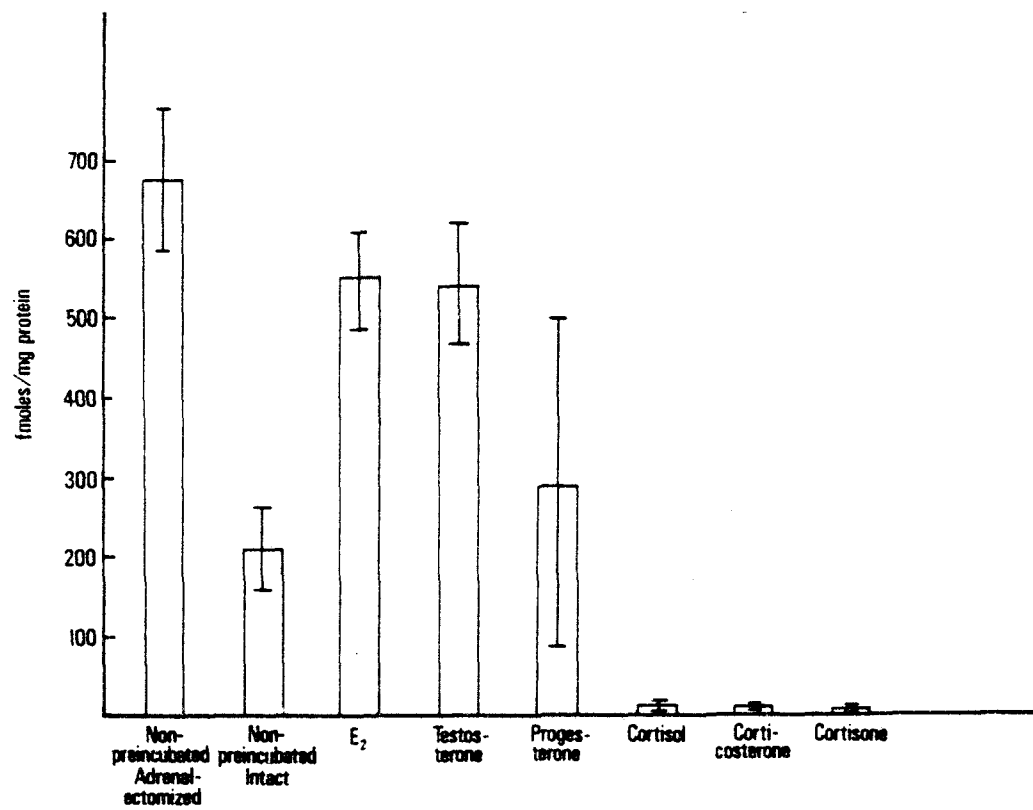


Figure 3
Marcusen and Rhees

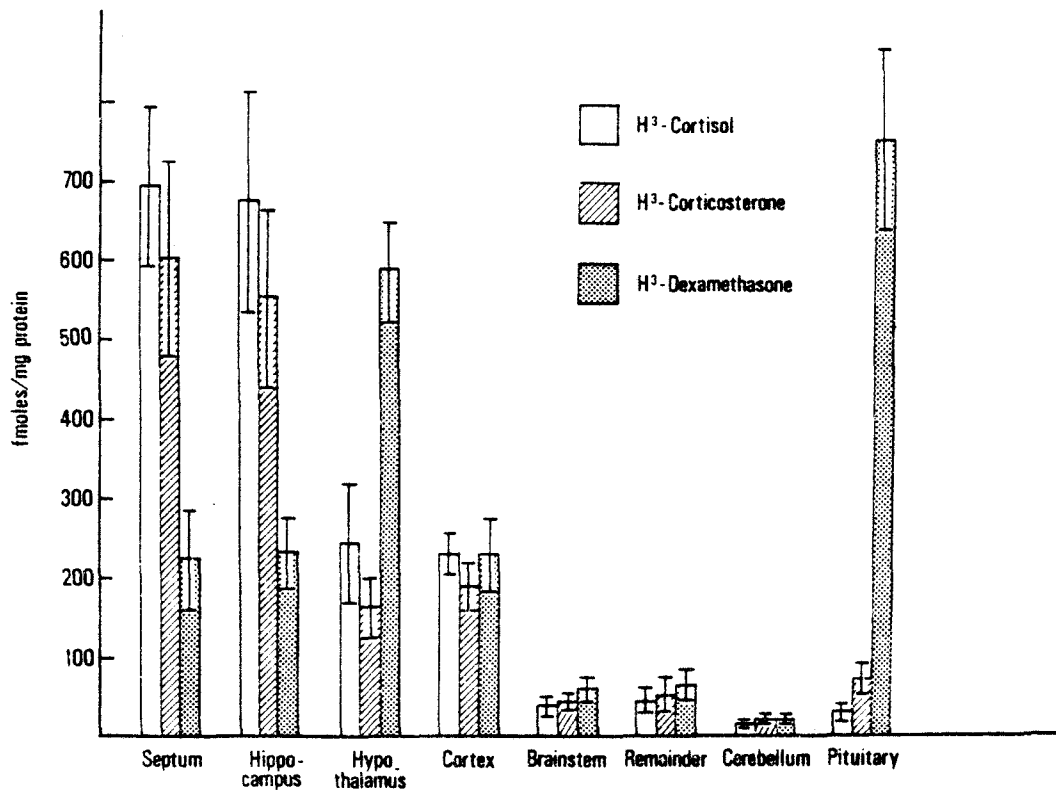


Figure 4
Marcusen and Rhees

CYTOSOL BINDING OF STEROID HORMONES IN
SHEEP BRAINS AND PITUITARIES

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ABSTRACT

[³H]-cortisol and [³H]-corticosterone were bound preferentially by cytosols from the hippocampal and septal regions of brains of adrenalectomized sheep, whereas, [³H]-dexamethasone was preferentially bound by cytosol from the pituitaries of the same animals. Cytosols isolated from the brains of adrenalectomized sheep bound significantly more [³H]-corticosterone than did cytosols from the brains of intact animals. These findings provide evidence that the sites of feedback inhibition are the same in the sheep as described for other mammalian systems, and that in the sheep as in other mammals the principle site of localization of cortisol and corticosterone is in limbic structures of the brain, whereas, the principle site of uptake of dexamethasone is in the pituitary.

COMMITTEE APPROVAL: _____

19 Mar 77

Date