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CHARACTERISTICS OF DEXAMETHASONE BINDING TO HIPPOCAMPAL CYTOSOL RECEPTORS

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SUMMARY

This study was designed to clarify an *in vitro* system which could be used to study the time course, kinetics, and specificity of [^{3}H]-dexamethasone (DM) binding in hippocampal cytosol. Maximal binding, as measured by chromatography through Sephadex LH-20 mini-columns, was attained by 6 h and maintained to 24 h when the incubation medium contained 20 mM Tris, 20% glycerol, 50 mM KCl, 20 mM monothioglycerol, pH 7.8. Cytosol from adrenalectomized pigs exhibited a 50% greater capacity to specifically bind DM than that from unadrenalectomized pigs. Non-specifically bound DM averaged 18 ± 5% of total [^{3}H]-DM bound. The calculated K_D was 1.07×10^{-10} M and the maximum number of binding sites was 3.0×10^{-14} moles/mg protein. Thirty-three hours were required for the dissociation of half of the specifically bound [^{3}H]-DM. Instability of the complex increased as incubation temperature was raised. Binding activity was diminished by competition with glucocorticoid or anti-glucocorticoid hormones and by incubation with proteolytic enzymes. It was demonstrated that DM binding in porcine serum is limited and that possible contamination of this system with CBG had negligible effects. Thus a glucocorticoid receptor exists in porcine hippocampal cytosol and can be characterized by its high affinity and specificity as different from CBG.

INTRODUCTION

The hippocampus has been referred to as a "window through which glucocorticoids modulate the activity of the central nervous system" [1]. Studies of glucocorticoid binding in various brain regions of duck [2], rat [3], pig [4] and monkey [5] have demonstrated that the hippocampus is the primary glucocorticoid-concentrating region of the brain. The effects of glucocorticoids on neurological function have been found to be manifested in a number of ways. The work of Endroczi[6] which demonstrated a correlation of specific glucocorticoid binding to subcellular components of hippocampal cells with the modulation of a behavioral parameter, along with similar studies by Nyakas and Endroczi[7] and Bohus and deKloet[8] provide compelling evidence for the hippocampus as a mediator of glucocorticoid effects upon the brain. Cortisol has been demonstrated to increase and prolong electrically-induced hippocampal seizure activity [9, 10]. This effect appears to be specific to the hippocampus [11]. Furthermore, several studies have implicated the hippocampus in the negative feedback inhibition of hydrocortisone on CRF-ACTH release, but the nature of that involvement remains obscure [12–14]. These data and others in the current literature support the concept of a relationship between glucocorticoid action upon the hippocampus and the functions of the hippocampus within the central nervous system.

Since the hippocampus has been shown to be one of the major brain regions possessing a mechanism for glucocorticoid retention [4, 15, 16], these experiments were purposed to investigate dexamethasone binding to receptors in hippocampal cell cytosol by examining certain features of that binding reaction *in vitro*.

EXPERIMENTAL PROCEDURE

Animals and tissues. Young pigs of mixed breeds, weighing 15–20 kg, were obtained from Wilson Hog Research Farms. Adrenalectomies were performed according to the method of Lustgarten *et al.*[17]. After recovery from surgery, the pigs were maintained on 0.9% saline and standard laboratory chow *ad libitum* until used experimentally (3–5 days post-surgery). Fresh brain tissues were also obtained from local slaughterhouse within minutes of death and the tissues were immediately placed in ice-cold buffer.

Chemicals. [1,2-^{3}H]-Hydrocortisone ([^{3}H]-F) (specific activity (S.A.) = 40 mCi/mmol), and [6,7-^{3}H]-dexamethasone ([^{3}H]-DM) (S.A. = 38 mCi/mmol) were purchased from New England Nuclear Corp. Non-labeled steroids were purchased from Sigma Chemical Co. except for cortexolone (Aldrich). Trypsin, type IX from hog pancreas, ribonuclease-A, type 1-A from bovine pancreas, deoxyribonuclease I, from bovine pancreas, neuraminidase, type VI from *Clostridium perfringens*, and protease were purchased from Sigma Chemical Co.

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Incubation conditions. The dissected hippocampi were minced in 2 volumes of ice-cold (20 mM Tris–HCl, 20% glycerol (V/V), 50 mM KCl, 20 mM monothioglycerol, pH 7.8) buffer, unless specifically stated in the text. All procedures were carried out between 0° and 3.5°C unless otherwise specified. The minced tissues were homogenized in a glass vessel with a Teflon pestle, and centrifuged at 10,000 *g* for 10 min. The supernatant was decanted and centrifuged at 150,000 *g* for 50 min to obtain the cytosol. Cytosol was diluted so that the final concentration of protein in the incubation was between 0.8 and 1.2 mg/ml.

Binding studies. The time course of binding was studied by incubation of cytosol with labeled DM with and without 100-fold excess of non-labeled DM for 30, 60, 120, 240, 360, 720 and 1440 min. The time course of dissociation of the steroid–receptor complex was investigated by the addition of 100-fold excess unlabeled DM to tubes pre-incubated with labeled DM for 18 h. In all cases, binding reactions were stopped by addition of the sample to Sephadex columns.

In other incubations, aliquots of cytosol were incubated with [^{3}H]-DM ranging in concentration from 8×10^{-8} M to 5×10^{-11} M. Parallel incubations were carried out with the addition of 2.5×10^{-6} M non-labeled DM. Data were analyzed by the method of Scatchard[18].

Competition experiments were performed by incubating aliquots of cytosol with 2×10^{-8} M [^{3}H]-DM with and without 2×10^{-6} M non-labeled steroid: dexamethasone, dexamethasone sodium phosphate, hydrocortisone, cortexolone, prednisolone, corticosterone, testosterone, estradiol, or progesterone.

The method of Lee *et al.*[19] was employed to study the effect of various enzymes on the binding reaction. Aliquots of cytosol were incubated with 2×10^{-8} M [^{3}H]-DM for 18 h at 3.5°C with and without the different enzyme solutions (1.0 mg/ml). After 18 h the amount of specifically bound hormone was determined.

Porcine serum was diluted with 20 mM Tris, 50 mM KCl, 10 mM Monothioglycerol, 20% glycerol, pH 7.8, so that the final protein concentration was 1.0 mg/ml. Aliquots of serum and hippocampal cytosol were incubated with 5×10^{-8} M [^{3}H]-DM or [^{3}H]-hydrocortisone (F) with and without 5×10^{-6} M DM or F for 24 h. Free hormone was separated from bound by Sephadex gel filtration.

Analyses. In all incubations, two series of tubes were prepared. In one series, each tube contained a known concentration of [^{3}H]-DM only. In the other series, each tube contained the same concentration of [^{3}H]-DM as its companion, plus a 100-fold excess of non-labeled DM. The amount of radiolabeled steroid bound to cytosol receptors was measured by gel filtration through Sephadex LH-20 (Pharmacia) columns (0.8 × 8.0 cm). The amount of hormone specifically bound in cytosol was determined by subtracting the amount of radioactivity bound in the tubes containing [^{3}H]-DM plus excess DM from the amount of hormone bound in tubes containing only [^{3}H]-DM.

Protein concentrations were determined in each case by the method of Lowry *et al.*[20], with corrections for interfering sulfhydryl reagents and potassium ions according to the method of Vallejo and Lagunas[21]. Samples were counted for 10 min in 10 ml of scintillation fluid containing 4.53 gm 2,5-

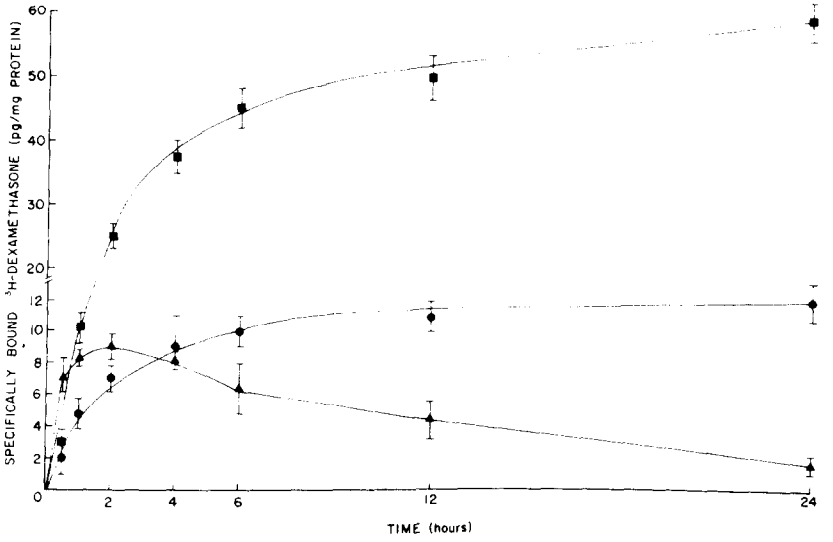

Fig. 1. Time course of specific [^{3}H]-DM binding in hippocampal cytosol in various buffers. Hippocampi were homogenized in 20 mM Tris, 20% glycerol, pH 7.8 (TG), ▲; 20 mM Tris, 20% glycerol, 50 mM KCl, 10 mM monothioglycerol, pH 7.8 ($TGT_{10}K$), ●; or 20 mM Tris, 20% glycerol, 50 mM KCl, 20 mM monothioglycerol, pH 7.8 ($TGT_{20}K$), ■. Tubes containing hippocampus cytosol were incubated with 2×10^{-8} M [^{3}H]-DM ± 2×10^{-6} M DM at 3.5°C. Binding was determined by chromatography on Sephadex LH-20 mini-columns. Non-specific binding was 18 ± 5% of total binding. Each point is the mean ± S.D. of 5 separate experiments.

diphenyl-oxazole (PPO), 0.057 gm, 1,4-bis-(5-phenyloxazoyl-2-)-benzene (POPOP) and 100 ml Biosolve BBS-3 (Beckman) in 1 l. of toluene. Counting efficiency was 49–51%. Quenching was monitored by the external standard-channels ratio method.

The variation between sample means was analyzed by an analysis of variance F test [22]. Multiple comparisons were performed using the Student–Newman–Keuls test [23]. Linear regression analysis was used for determination of the slope of linear plots. Significance was tested at the 0.05 level. For figures and tables the means and standard deviations (S.D.) are recorded.

RESULTS

When cytosol was prepared in 20 mM Tris, 20% glycerol, pH 7.8 (TG) buffer, the greatest amount of specifically bound hormone was measured at 2 h of incubation at 3.5°C. After 2 h, specific binding activity decreased so that less than 25% of maximal binding was present after 24 h (Fig. 1). The addition of 50 mM KCl and 10 mM monothioglycerol to the homogenization buffer ($TGT_{10}K$) resulted in a 33% increase in the maximum amount of labeled hormone specifically bound. The amount specifically bound began to plateau between 6 and 12 h of incubation time. The reports of Granberg and Ballard[24] and of Schrader (personal communication) suggested that increasing the monothioglycerol concentration to 20 mM would result in an increased stability of rat brain glucocorticoid receptors. Figure 1 also depicts the results from time course experiments in which 20 mM Tris, 20% glycerol, 50 mM KCl, 20 mM monothioglycerol, pH 7.8 ($TGT_{20}K$) buffer was employed. The maximal amount of specific binding in these experiments was 5 times greater than that when $TGT_{10}K$ buffer was used. A plateau of maximal binding was reached by about 6 h of incubation at 3.5°C and was maintained out to 24 h of incubation. Therefore, $TGT_{20}K$ buffer was utilized in the remainder of experiments and an incubation time of 18 h was chosen to insure that equilibrium conditions for the binding reaction were being approached.

Kinetics of binding

Hippocampal cytosol was incubated with various concentrations of [^{3}H]-dexamethasone ([^{3}H]-DM) at 3.5°C for 18 h. As Fig. 2 illustrates, the binding reaction reached a plateau of maximal binding, indicating saturation of specific binding sites, at about 2×10^{-8} M [^{3}H]-DM. The % of total [^{3}H]-DM measured as non-specifically bound averaged 18 ± 5%. The amount of specifically bound hormone in cytosol isolated from adrenalectomized pigs (ADX) was 50% greater than that from unadrenalectomized pigs. When the binding data from the ADX pigs were plotted according to the method of Scatchard[18], Fig. 3 was generated. The K_D calculated by this method was 1.07×10^{-10} M and n, the maximum number of binding sites, was 3.0×10^{-14} mol/mg protein.

Figure 4 depicts the reversibility of the binding reaction. Sixty-five % of the specifically bound [^{3}H]-DM was dissociated after 48 h at 3.5°C. The half-time of dissociation was 33 h, as calculated by linear regression analysis. It therefore required 33 h

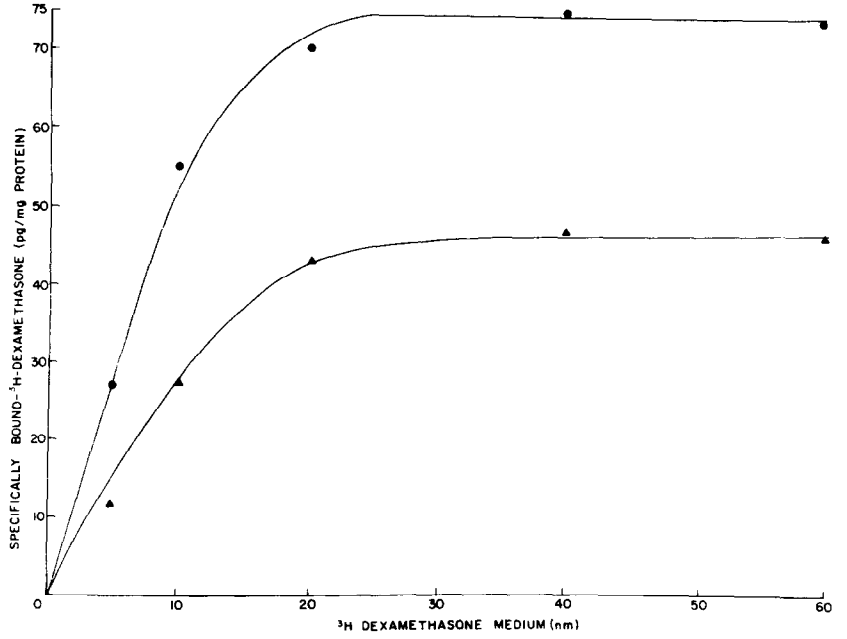

Fig. 2. Concentration-dependence of specific [^{3}H]-DM binding in hippocampal cytosol. Hippocampi isolated from intact pigs, ▲, and adrenalectomized pigs, ●, were homogenized in $TGT_{20}K$ and incubated for 18 h at 3.5°C with concentrations of [^{3}H]-DM from 0.05×10^{-8} M to 6×10^{-8} M ± 2.5×10^{-6} M DM. Each point represents the mean of 4 separate experiments.

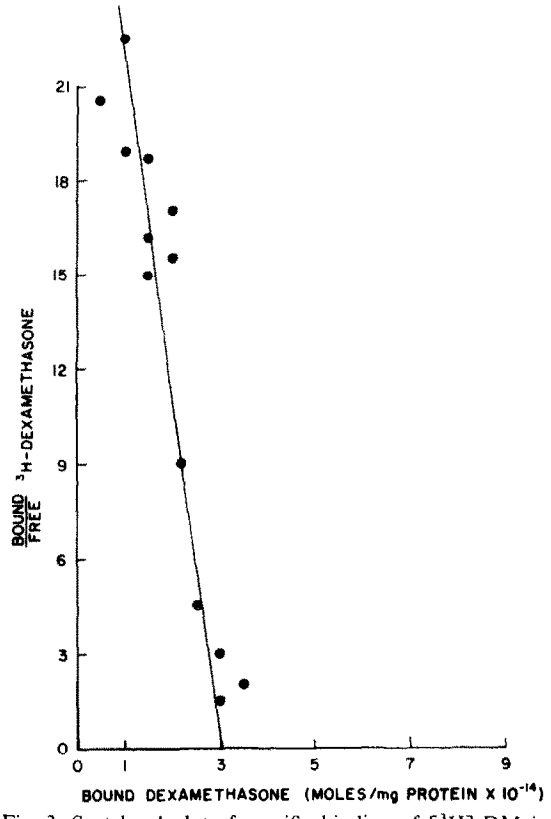

Fig. 3. Scatchard plot of specific binding of [^{3}H]-DM in hippocampal cytosol isolated from adrenalectomized pigs.

for half of the specifically bound [^{3}H]-DM to dissociate from cytosol binding sites, under the conditions employed.

When hippocampal cytosol was incubated for 18 h at 3.5°C with 2×10^{-8} M [^{3}H]-DM plus or minus 2×10^{-6} M DM and then incubated an additional 2 h at 3.5, 22 or 37°C, the data in Fig. 5 resulted. Bound hormone complexes remained stable at 3.5°C, but rapidly dissociated at 22 or 37°C. Sixty % of specifically bound hormone was dissociated after 2 h at 22°C, whereas all specifically bound hormone was dissociated after 1 h of incubation at 37°C.

Specificity of binding

Table 1 summarizes the results of experiments wherein 2×10^{-8} M [^{3}H]-DM was incubated with hippocampal cytosol in the presence or absence of 2×10^{-6} M non-labeled steroids. Non-radioactive DM was the best competitor, followed by two steroids with anti-glucocorticoid activity, progesterone and cortexolone, with estradiol-17β and testosterone the least effective inhibitors. These data also indicate that the receptor binds to steroids with glucocorticoid or anti-glucocorticoid activity but not to the sex steroids, testosterone or estradiol-17β. Data from competition experiments utilizing unlabeled DM compared to those utilizing unlabeled dexamethasone sodium phosphate demonstrate that steroid solubility was not a significant variable.

Enzyme degradation of binding activity

A preliminary determination of the types of biochemical moieties which must be present in the receptor molecule to exhibit glucocorticoid binding activity was investigated by incubating cytosol in the presence of different enzymes (Table 2). The action of proteolytic enzymes, protease and trypsin, resulted in the greatest loss of bound hormone after an 18 h incubation at 3.5°C. Deoxyribonuclease and neuraminidase had no significant effects on binding activity. RNase treatment caused a slight reduction that was statistically significant. These results indicate that the receptor is largely protein and may have ribonucleotide moieties, as well.

Receptor and serum binding

A comparison was made between binding of [^{3}H]-DM and [^{3}H]-hydrocortisone (F) to hippocam-

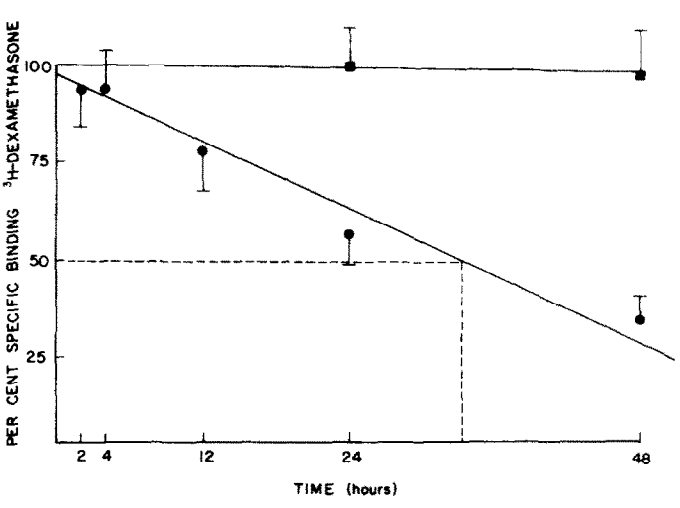

Fig. 4. Demonstration of reversibility of [^{3}H]-DM binding. Hippocampal cytosol was incubated as described in Experimental Procedure. After 18 h, 2×10^{-6} M DM was added to the tubes containing cytosol plus 2×10^{-8} M [^{3}H]-DM. Specific binding was determined at 2, 4, 12, 24 and 48 h from the time of addition of DM. ■, undissociated specific binding; ●, binding after dissociation.

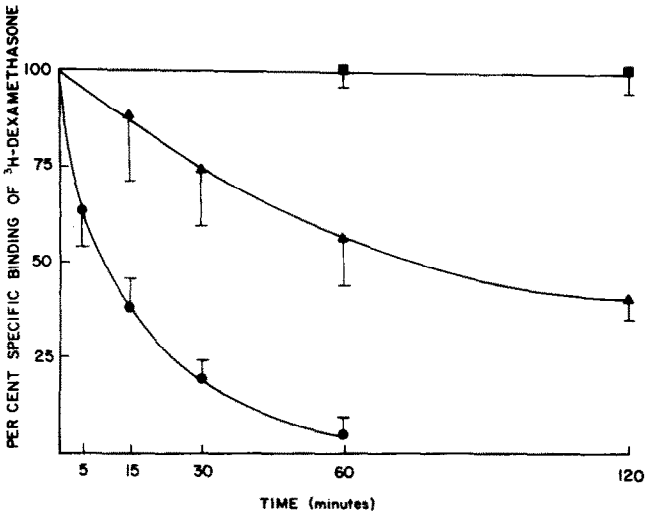

Fig. 5. Temperature-dependence of specific [^{3}H]-DM binding. Hippocampal cytosol was incubated as described in the text for 18 h at 3.5°C and for an additional 2 h at 3.5°, ■, 22°, ▲, and 37°, ●. Data are expressed as per cent of control. Each point represents the mean ± S.D. of 6 determinations.

pal cytosol receptors and hog serum glucocorticoid binders (Table 3). Eighty times more [^{3}H]-DM was specifically bound in cytosol than in serum, whereas 280 times more [^{3}H]-F was specifically bound in serum than in cytosol. Dexamethasone is an effective competitor of [^{3}H]-F binding to cytosol proteins, but non-radioactive F is the better competitor for binding to serum binders. Since the extent of [^{3}H]-DM interaction with serum binding sites is extremely small, competition of the unlabeled steroids with [^{3}H]-DM for serum binding sites was not demonstrable. These results clearly indicate that there are significant differences in the ability of natural and synthetic glucocorticoids to bind porcine serum receptors compared to hippocampal cytosol. Therefore, the degree of error introduced into the cytosol binding experiments by the presence of CBG was very small and negligible.

DISCUSSION

Preparation of hippocampal cytosol in a buffer containing 20% glycerol, 20 mM monothioglycerol and 50 mM KCl permitted an environment in which the greatest magnitude of binding could be measured and in which peak binding activity could be maintained out to 24 h of incubation at 3.5°C. Other investigators have previously discovered the advantages of such buffers. Granberg and Ballard[24] reported that the presence of monothioglycerol in the homogenization medium prevented oxidation of the receptor site sulfhydryl groups and resulted in increased receptor stability. Krieger *et al.*[25] demonstrated that maximal stability of free receptors required 50 mM KCl in the homogenization buffer, since binding activity was rapidly lost at higher or lower ionic strengths. Goral and Wittliff[26] reported a two-fold increase in specific glucocorticoid binding by adding monothioglycerol to the homogenization medium. Increased stability of the glucocorticoid receptor has also been reported by Schmid *et al.*[27] after the addition of 50 mM KCl and sulfhydryl reagents. It is not possible on the basis of data presented to define the mechanism whereby binding activity is increased and maintained in this buffer system. Enhanced association, decreased dissociation, stabilization of receptor or

Table 1. Specificity of [^{3}H]-dexamethasone binding for hippocampal cytosol

Competitor	Per cent reduction of binding (±S.D.)
Dexamethasone	100 ± 3
Dexamethasone sodium phosphate	100 ± 5
Progesterone	72 ± 4
Cortexolone	68 ± 6
Cortisol	39 ± 7
Corticosterone	35 ± 7
Estradiol-17-beta	5 ± 6
Testosterone	2 ± 3

Cytosol from pooled slaughterhouse hippocampi was incubated with 2×10^{-8} M [^{3}H]-DM with and without 2×10^{-6} M nonlabelled steroid for 8 h at 3.5°C. The data are expressed as per cent reduction in [^{3}H]-DM binding relative to dexamethasone ($n = 5$).

Table 2. Effects of Enzymes on [^{3}H]-dexamethasone binding

Enzyme	Amount bound (pg [^{3}H]-dexamethasone per mg cytosol protein)
None	57.96 ± 2.05
Protease	8.94 ± 2.62
Trypsin	26.49 ± 6.12
RNase	42.89 ± 3.61
Neuraminidase	51.23 ± 3.28
DNase	59.97 ± 10.34

Cytosol isolated from intact pig hippocampi was incubated with 2×10^{-8} M [^{3}H]-DM with and without 2×10^{-6} M nonlabelled DM for 18 h at 3.5°C. Enzyme treatment involved incubating in the presence of 1 mg per ml of different enzymes ($n = 6$). Values are means ± S.D.

Table 3. Comparison of [^{3}H]-dexamethasone (DM) and [^{3}H]-hydrocortisone (F) binding in serum and in cytosol. Amount bound (pg [^{3}H]-steroid per mg cytosol protein)

	[^{3}H]-DM	[^{3}H]-DM + DM	[^{3}H]-DM + F	[^{3}H]-F	[^{3}H]-F + DM	[^{3}H]-F + F
Cytosol	17.65	6.29	9.68	1.67	0.61	0.78
	±0.78	±0.32	±0.65	±0.16	±0.05	±0.05
Serum	0.69	0.55	0.56	257	233	8.27
	±0.08	±0.03	±0.02	±14.4	±30.0	±1.34

Hippocampal cytosol and serum were isolated from slaughterhouse animals and incubated with 2×10^{-8} M [^{3}H]-DM or [^{3}H]-F in the presence or absence of 2×10^{-6} M DM or F. Incubations were carried out at 3.5°C for 24 h ($n = 5$). Values are means ± S.D.

steroid–receptor complexes, and inhibition of degradation could each be involved. It is important for this study that an *in vitro* system was used which yielded maximum binding activity and a time course of binding activity from which could be determined approximation of binding equilibrium for subsequent kinetic studies.

Scatchard analysis revealed a class of high affinity sites for DM. Our calculated value for K_D at 3.5°C of 1.07×10^{-10} M is within the general range of values reported by others for DM binding to rat hippocampal cytosol [16, 28] and bovine pituitary cytosol [29]. The slightly smaller value obtained from the present studies may be related to the presence of monothioglycerol in the medium. Granberg and Ballard[24] also reported a value for K_D that was smaller when dithiothreitol was present than in its absence. That the receptor does not readily dissociate from its bound ligand is also demonstrated by the slow reversibility of binding. Thirty-three h were required to dissociate 50% of the bound hormone. This time course is similar to that reported by Schaumberg[30] and Pratt, *et al.*[31] for other glucocorticoid systems. It should be pointed out that although these data were obtained in an ambient temperature (3°C) at which the steroid binder is stable, the kinetic and specificity data may not be the same at 37°C.

Although synthetic glucocorticoids are 10–100-fold more active than naturally occurring adrenal steroid hormones at equimolar concentrations, it is generally accepted that both types bind to the same cytoplasmic receptor prior to the initiation of an organ specific process. However, it is difficult to reconcile these and other data on stereospecificity with a simple competitive inhibition model. Agarwal[32] reported that synthetic steroid–receptor complexes migrated differently than complexes possessing natural corticoids in a column chromatographic system. He concluded that natural and synthetic steroid hormones bind to physico-chemically distinct receptor proteins. DeKloet and McEwen[16] also observed more than one population of specific glucocorticoid binding sites with overlapping specificity and different localization. Dexamethasone and corticosterone had different patterns of cytoplasmic binding in various brain regions. Cytoplasmic binding of dexamethasone in the hippocampus was slightly greater than corticosterone, but the latter bound to hippocampal cell nuclei to a greater extent than dexamethasone. Furthermore, dexamethasone was not as good a competitor for [^{3}H]-corticosterone as corticosterone. The authors alluded to the fact that different corticoids have differential effects on behavior not commensurate with their glucocorticoid activities. For example, corticosterone has been shown to decrease passive avoidance behavior after implantation in the hippocampus, whereas a synthetic corticoid did not have the same effect. The conclusion of deKloet and McEwen[16] was that binding of corticoids in the hippocampus may involve a stereospecificity more related to behavioral effects than to glucocorticoid activity. Furthermore, the presence of more than one population of corticoid binding sites in the hippocampus cannot be excluded.

Since the brain tissues used in this study were not perfused *in situ*, the question arises as to the extent of contamination of the system by plasma dexamethasone-binding proteins. We have shown, however, that the binding of this steroid to blood-borne receptors is only 1.2% of that in the hippocampus cytosol. Since the binding of hydrocortisone to plasma receptors is significantly greater than that of DM, the finding that [^{3}H]-DM binding to cytosol receptors was 10 times greater than that of [^{3}H]-F further indicates the absence of a significant amount of blood contamination of cytosol preparations.

These studies have successfully utilized an *in vitro* system to elucidate some of the characteristics of specific binding of DM to receptors in hippocampus cytosol of the pig. It is important to understand these characteristics because the level of adrenocortical activity registered in the hippocampus, via a receptor mechanism, may be found to play an important role in the modulation of hippocampal function.

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REFERENCES

1. Nakajima S.: Hippocampal protein synthesis and spike discharges in relation to memory; In *The Hippocampus* (Edited by Isaacson and Pribram). Plenum, New York (1975), Vol. 1, pp. 393–413.

2. Rhees R. W., Abel J. H. Jr and Haack D. W.: Uptake of tritiated steroids in the brain of the duck (*Anas platyrhynchos*): an autoradiographic study. *Gen. comp. Endocr.* **18** (1972) 293–300.
3. Warembourg M.: Radioautographic study of the rat brain after injection of [1,2-^{3}H]-corticosterone. *Brain Res.* **89** (1975) 61–70.
4. Stith R. D., Person R. J. and Dana R. C.: Uptake and binding of [^{3}H]-hydrocortisone by various pig brain regions. *Brain Res.* **117** (1976) 115–124.
5. Pfaff D. W., Gerlach J. L., McEwen B. S., Ferin M., Carmel P. and Zimmerman E. A.: Autoradiographic localization of hormone-concentrating cells in the brain of the female Rhesus monkey. *J. comp. Neurol.* **170** (1976) 279–294.
6. Endroczi E.: Mechanism of steroid hormone actions on motivated behavioral reactions; In *Hormones, Homeostasis, and the Brain, Recent Progress in Brain Research* (Edited by Gispen, van Wimersma Greidanus, Bohus and De Weid) Vol. 42 Elsevier, Amsterdam (1975) pp. 125–134.
7. Nyakas C. and Endroczi E.: Neonatal effects of corticosterone on adult behavior and the uptake of labelled corticosterone by hippocampus. *Acta endocr Copenh., Supp.* **199** (1975) 137, Abstract No. 74.
8. Bohus B. and DeKloet E. R.: Behavioral effects of corticosterone related to glucocorticoid receptor properties in the rat brain. *J. Endocr.* **72** (1976) 64P–65P.
9. Conforti N. and Feldman S.: Effect of cortisol on the excitability of limbic structures of the brain in freely moving rats. *J. Neuro. Sci.* **26** (1975) 29–38.
10. Endroczi E. and Lissak K.: Interrelations between paleocortical activity and pituitary-adrenal function. *Acta Physiol. Hung.* **21** (1962) 257–263.
11. Conforti N. and Feldman S.: Effects of dorsal fornix section and hippocampectomy on adrenocortical responses to sensory stimulation in the rat. *Neuroendocrinology* **22** (1976) 1–7.
12. Stith R. D., Person R. J. and Dana R. C.: Effects of hippocampal and amygdalar stimulation on uptake and binding of [^{3}H]-hydrocortisone in the hypothalamus of the cat. *J. Neurosci. Res.* **2** (1976) 317–322.
13. Kawakami M., Seto K., Terasawa E., Yoshida K., Miyamoto T., Sekiguchi M. and Hattori, Y.: Influence of electrical stimulation or lesion in limbic structure upon biosynthesis of adrenocorticoids in the rabbit. *Neuroendocrinology* **3** (1968) 337–348.
14. Davidson J. M. and Feldman S.: Effects of extrahypothalamic dexamethasone implants on the pituitary-adrenal system. *Acta endocr. Copenh.* **55** (1967) 240–246.
15. DeKloet R. and McEwen B. S.: In *Molecular and Functional Neurobiology* (Edited by W. H. Gispen). Elsevier, Amsterdam (1976) pp. 257–307.
16. De Kloet E. R., Wallach G. and McEwen B. S.: Differences in corticosterone and dexamethasone binding to rat brain and pituitary. *Endocrinology* **96** (1975) 598–609.
17. Lustgarten C., Bottoms G. D. and Shaskas J. R.: Experimental adrenalectomy of pigs. *Am. J. Vet. Res.* **34** (1973) 279–282.
18. Scatchard G.: The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51** (1949) 660–672.
19. Lee D. K. H., Stern M. and Solomon S.: Cytoplasmic glucocorticoid receptors in the developing small intestine of the rabbit fetus. *Endocrinology* **99** (1976) 379–388.
20. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: Protein measurement with folin phenol reagent. *J. biol. Chem.* **193** (1951) 265–275.
21. Vallejo C. G. and Lagunas R.: Interference by sulfhydryl, disulfide reagents and potassium ions on protein determination by Lowry's method. *Analyt. Biochem.* **36** (1970) 207–212.
22. Klugh H. E.: *Statistics, the Essentials for Research.* Wiley, New York (1970) pp. 210–238.
23. Zar J. H.: *Biostatistical Analysis.* Prentice-Hall, Englewood Cliffs (1974) pp. 151–162.
24. Granberg J. R. and Ballard P. L.: The role of sulfhydryl groups in the binding of glucocorticoids by cytoplasmic receptors of lung and other mammalian tissues. *Endocrinology* **100** (1977) 1160–1168.
25. Krieger N. S., Middlebrook J. L. and Aronow L.: Effect of salt on reversibility of glucocorticoid receptor binding. *J. steroid Biochem.* **7** (1976) 395–399.
26. Goral J. E. and Wittliff J. L.: Comparison of glucocorticoid-binding proteins in normal and neoplastic mammary tissues of the rat. *Biochemistry* **14** (1975) 2944–2952.
27. Schmid H., Grote H. and Sekeris C. E.: Stabilization and characterization of the dexamethasone-binding proteins in rat liver cytosol. *Molec. cell Endocr.* **5** (1976) 223–241.
28. Koch B., Lutz B., Briaud B. and Mialke C.: Glucocorticoid binding to adenohypophysis receptors and its physiological role. *Neuroendocrinology* **18** (1975) 299–310.
29. Watanabe H.: Dexamethasone-binding receptor in bovine pituitary cytosol. *J. steroid Biochem.* **6** (1975) 27–33.
30. Schaumberg B.: Investigations on the glucocorticoid-binding protein from rat thymocytes—II. stability, kinetics and specificity of steroids. *Biochim. biophys. Acta* **261** (1972) 219–235.
31. Pratt W. B., Kaine J. L. and Pratt D. V.: The kinetics of glucocorticoid binding to the soluble specific binding proteins of mouse fibroblast. *J. biol. Chem.* **250** (1975) 4584–4591.
32. Agarwal M. K.: Evidence that natural vs synthetic steroid hormones bind to physiochemically distinct cellular receptors. *Biochem. biophys. Res. Commun.* **73** (1976) 767–772.