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THE RELEASE OF CORTICOSTERONE AND A CORTICOSTERONE-BINDING PROTEIN BY INCUBATED RAT ADRENAL SLICES

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Summary—Stimulation of incubated rat adrenal slices with ACTH(1–24) resulted in an increase in the release of both corticosterone and specific corticosterone-binding protein into the incubation medium. The release of corticosterone and binding protein was dose and calcium dependent with adrenals from animals pretreated with betamethasone. While the secretion of corticosterone was continuous throughout the incubation period, there appeared to be a limit to the increase in binding capacity. The specificity of steroid binding to the adrenal protein showed a similar profile to that of corticosteroid-binding globulin (CBG) in rat serum. A Western blot analysis using anti-rat CBG as the primary antiserum, showed that the adrenal protein was not CBG. [^{3}H]corticosterone binding with disc electrophoresis, run at 2°C, gave a single peak with approximately the same R_f value for rat serum, purified CBG, and adrenal incubate; at 22°C peaks were only seen for rat serum or purified CBG. The data presented provides further evidence for the existence of a specific corticosterone-binding protein of adrenal origin released in conjunction with corticosterone. The adrenal protein would appear to have a lower affinity for corticosterone than does CBG, and to be functionally more labile. It is possible that the adrenal protein may be CBG that has been internalized, modified and released with corticosterone.

INTRODUCTION

Corticosteroid-binding globulin (CBG), the high affinity binding protein for glucocorticoids in plasma, is known to be synthesized in the liver [1]. However, proteins with steroid-binding affinities similar to those of the circulating binding protein have been found in the adrenal cortex [2, 3]. While the function of these proteins is uncertain, there is evidence that they may be involved in intracellular storage and secretion of the steroid hormones from the adrenal cells; the steroid-binding proteins being released in conjunction with the glucocorticoids. Rubin *et al.* [4] found that ACTH (adrenocorticotrophic hormone) not only caused the release of corticosteroids, but also induced the release of protein; both steroid and protein release being calcium dependent. Pritchett *et al.* [5] found that the administration of ACTH, *in vivo* or *in vitro*, resulted in the release of an intraglandular corticosterone-binding ligand from incubated adrenal glands. Scatchard analysis of the corticosterone-binding activity in the adrenal incubation medium indicated the presence of a single binding protein which was different from either CBG or cytoplasmic glucocorticoid receptor proteins [6].

More recently, Bassett [7] showed that exposure of rats to a stressor produced a significant increase in both plasma corticosterone concentration and specific binding capacity. On the basis of the data obtained it was hypothesized that there was a specific CBG-like protein of adrenal origin released in conjunction with corticosterone; this binding protein having a lower affinity for corticosterone and a shorter functional half-life than CBG.

The present study was undertaken to investigate such a simultaneous release of steroid and binding protein, using incubated adrenal slices.

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Abbreviations used: CBG, corticosteroid-binding globulin; ACTH, adrenocorticotrophic hormone; DCC, dextran-coated charcoal; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; TBS, Tris buffered saline; K_d, apparent dissociation constant; M_W, molecular weight.

EXPERIMENTAL

Animals

Experimentally naive male CSF rats (90 ± 5 days-old) were used in all experiments. The animals were housed in groups of 3 under

conditions of constant temperature and humidity (21 ± 0.5°C, 46% humidity) and subjected to a 12 h reversed light–darkness schedule (lights on from 20.00 to 08.00 h), beginning at least 14 days before the commencement of experimentation and continuing until its conclusion. Food and water were available *ad libitum*.

Betamethasone pretreatment

Animals which were pretreated with betamethasone (9α-fluoro-16β-methyl-11β, 17α, 21-trihydroxy-1,4-pregnadiene-3,20-dione; Sigma Chemical Co., St Louis, MO, U.S.A.) were given the synthetic steroid in their drinking water (50 μmol/l) for 24 h before the commencement of the experiment. Betamethasone was given in order to suppress endogenous ACTH release from the anterior pituitary [8].

Incubation procedure

Animals were killed by cervical dislocation and exsanguination, and both adrenal glands rapidly removed and stored at 4°C. Three pairs of adrenals from either betamethasone pretreated or untreated animals were weighed, quartered, then placed in a stainless steel mesh basket (dia 12 mm; height 15 mm). The adrenal slices were rinsed in Krebs–Henseleit solution at 4°C before being placed in an organ bath containing Krebs–Henseleit solution maintained at 37°C and gassed with 5% CO_2 in oxygen and subjected to four 1 min washes.

Following the washing procedure, the adrenal slices were rinsed with Krebs solution then transferred to a 2nd organ bath containing 15 ml of Krebs solution to which was added ascorbic acid (40 μg/ml) and the proteinase inhibitor, aprotinin (Trasylol; Bayer Pharmaceutical Co. Leverkusen, Germany, 5000 kallikrein inactivator U/ml). The Krebs solution was maintained at 37°C and gassed as before. Where the slices were stimulated with ACTH(1–24), the peptide was added to the organ bath immediately prior to the adrenal slices. The nominal bath concentrations of ACTH(1–24) studied were 68 and 136 nM. Aliquots of the incubation medium were taken for either corticosterone or specific binding capacity analysis at 30 and 60 min.

Partition chromatography

Aliquots of incubation medium were taken and the steroids extracted with dichloromethane, then separated using partition chromatography on celite columns as described by Henry and Bassett [9]. Recovery studies using labelled steroids showed that 84.3 ± 4.3% and 96.8 ± 1.9% (mean ± SEM) of progesterone and deoxycorticosterone, respectively were removed with the 20% mobile phase, 88.3 ± 2.4% of corticosterone with the 30% mobile phase and 93.0 ± 4.3 of 18-hydroxycorticosterone eluting at >50%. The corticosterone fraction was evaporated to dryness under nitrogen and assayed for corticosterone. The corticosterone content of the incubation medium was corrected for incomplete recovery and loss during separation. The mean % recovery for all estimates was 88.3 ± 1.8%.

18-Hydroxydeoxycorticosterone was found to co-elute with corticosterone (87.3 ± 2.7% eluting in the 30% mobile phase), however this steroid did not bind to rat CBG, the binding protein used in the competitive protein-binding assay (see Scatchard analysis, Fig. 1).

Assay for corticosterone

The method used was the modified competitive protein-binding assay of Henry and Bassett [9] in which 1% rat plasma stripped with dextran-coated charcoal (DCC) (625 mg charcoal; Sigma Chemical Co., St Louis, MO, U.S.A.), 62 mg dextran T70 (Pharmacia

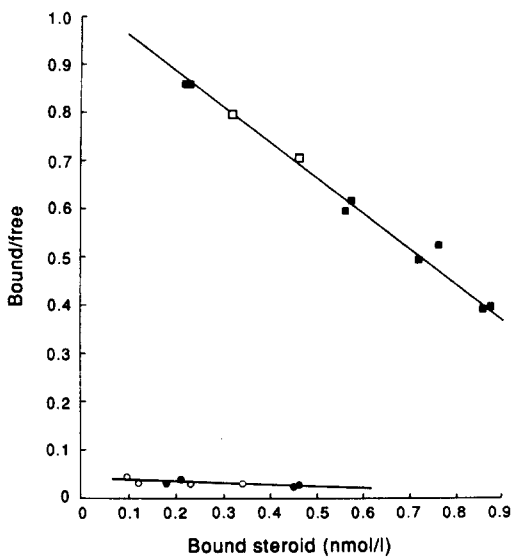

Fig. 1. Scatchard plot of [^{3}H]corticosterone (■) and [^{3}H]18-hydroxydeoxycorticosterone (●) binding to rat serum protein. Serum was stripped of endogenous steroids using DCC and then diluted to 0.1% with phosphate buffer. Diluted serum was then incubated with the labelled steroid for 30 min at 37°C followed by 15 min at 4°C. Unbound steroid was removed using DCC at 4°C. Linear regression analysis gave a regression coefficient of 0.994 and an apparent K_d 1.41 nmol/l for corticosterone. Open points indicate duplicate points. Almost identical results were observed when the experiments were repeated with 3 different rat sera.

Chemicals; Uppsala, Sweden), 100 ml phosphate buffer (pH 7.0) containing 30% glycerol was used as the binding protein. The inter- and intra-assay coefficients of variation were both $<5\%$.

Assay of binding capacity

Binding capacity was assayed by a slight modification of the procedure described by Ballard *et al.* [10] where prior separation of endogenous steroid is not required. Triplicate 1 ml aliquots of incubation medium were added to tubes containing 3.2 pmol of [^{3}H]corticosterone (46.5 Ci/mmol; Amersham, Bucks., U.K.), 36.3 nmol unlabelled corticosterone and 2 nmol of dexamethasone (9-fluoro-16α-methyl-11β,17α, 21-trihydroxy-1,4-pregnadiene-3, 20-dione; Sigma Chemical Co.) The unlabelled exogenous steroid was 8 times the maximum endogenous steroid determined in the incubation medium. This ratio of exogenous to endogenous steroid is in close agreement to that used by Ballard *et al.* [10] After vortexing the tubes were incubated for 30 min at 37°C followed by 1 h at 4°C. The unbound steroid was then removed using DCC at 4°C. An aliquot of the supernatant was transferred to scintillation fluid and counted in a Packard scintillation counter. A charcoal control (also in triplicate) in which the incubation medium was replaced by an equal volume of Krebs solution and subjected to all of the procedures described above was carried out for each adrenal incubate. Binding capacity was calculated as total corticosterone concentration (endogenous plus unlabelled exogenous plus [^{3}H]corticosterone) times the fraction of counts bound (incubation medium counts − charcoal control counts divided by total counts added). The results were expressed as pmol corticosterone bound/mg adrenal weight. In preliminary studies varying the amount of unlabelled exogenous corticosterone from 3.2 to 65.3 nmol indicated that corticosterone binding sites were saturated using 36.3 nmol of unlabelled corticosterone. The coefficient of intra-assay variation for the assay was 6%.

Addition of 6 μg albumin to the assay tubes (equivalent to approx. 1/5 of total protein released during incubation) did not alter the values obtained for the binding capacity. Ballard *et al.* [10] also reported that steroid binding to albumin was not detected in this assay. Charcoal treatment of either incubate or 0.1% plasma before assay, to remove endogenous corticosterone, dramatically reduced the binding capacity in the incubate but did not alter the values obtained for plasma (Table 1). A similar finding for plasma was reported by Ballard *et al.* [10].

Specificity of binding

Serum was stripped of endogenous steroids with DCC and diluted with buffer to a final concentration of 0.1%. For the adrenal incubate 2 lots of 5 pairs of adrenals were incubated as above, and stimulated with ACTH (1–24) (136.4 nM) for 15 min. The incubates were then pooled and stripped of steroids with DCC. Stripped serum or incubate was then incubated with 1.2 nmol/l of [1,2-^{3}H]corticosterone together with 1, 10 or 1000 times molar ratio of unlabelled competing steroid in the presence of 1000-fold dexamethasone, at 37°C for 30 min followed by 15 min at 4°C. Unbound steroid was removed using DCC at 4°C as described above. The results were expressed as % inhibition of [^{3}H]corticosterone bound in the absence of competing steroid. The competing steroids tested were aldosterone, testosterone, progesterone, deoxycorticosterone and corticosterone.

Electrophoresis and Western blot analysis

Serum, and incubate from 3 pairs of adrenals stimulated with ACTH(1–24) (136.4 nM) was diluted in sample buffer (62.5 mM Tris–HCl, pH 6.8; 10% v/v glycerol; 2% w/v SDS; 5% v/v 2-mercaptoethanol) and boiled for 5 min before electrophoresis.

Diluted serum, and an amount of incubation medium having twice the equivalent [^{3}H]corticosterone binding capacity, were then subjected to reducing SDS-polyacrylamide gel electrophoresis (PAGE) followed by Western blot analysis. Slab gel electrophoresis (10%) was carried out on a Bio-Rad mini-protein II cell apparatus using the buffer system of Laemmli [11] at 120V. The proteins were then

Table 1. The effect of prior stripping of endogenous corticosterone on the measurement of binding capacity (mean ± SEM; $n = 3$)

	Binding capacity	
	Non-stripped	Stripped
Incubate (pmol/mg tissue)	83.4 ± 7.4	3.3 ± 3.3
0.1% Plasma (pmol/ml plasma)	348 ± 21.0	366 ± 23.8

Endogenous steroid stripped using an equal volume of DCC at 37°C for 15 min. Non-stripped samples were diluted in a corresponding fashion using phosphate buffer.

transferred to nitrocellulose using the dry blot method of Kyhse–Andersen [12]. The nitrocellulose blots were washed twice in distilled water to remove SDS and incubated for 10 min in 10% skimmed milk. The nitrocellulose was then incubated for 1 h at room temperature with the primary monoclonal mouse anti-rat CBG (Ascites fluid antibody diluted 1:300 in TBS; 20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.5). After 3 × 10 min washes in TBS, the nitrocellulose was incubated for 1 h at room temperature with the secondary antibody (goat anti-mouse IgG conjugated with alkaline phosphotase; Bio-Rad, Australia) diluted 1:3000 in TBS. Excess secondary antibody was removed by TBS washes and CBG was visualized using the BCIP/NBT alkaline phosphotase colour development reagents (Bio-Rad, Australia).

Monoclonal antibodies

Four female Balb/c mice were immunized initially with 50 μg of purified rat CBG in complete Freund's adjuvant and incomplete Freund's thereafter at 3-week intervals for 3 months. Two weeks later the animal with the highest titre was injected with 60 μg CBG in water. Cell fusion (4 days after last injection) was essentially by the method of Oi and Herzenberg [13], with modifications. Spleen cells (10^8) were fused with 1×10^8 NS1 myeloma cells in 35% polyethelene glycol. Fusion products were distributed into six 96-well plates in selective medium. Strong positive cultures were expanded and cloned twice by limiting dilution and once by picking individual cells. Ascites fluid was produced by injecting 2×10^6 hybridoma cells into pristane primed Balb/c mice.

[^{3}H]corticosterone binding with disc electrophoresis

Serum was diluted 1:10 with sample buffer (62.5 nM Tris–HCl, pH 6.8; 10% glycerol; 1% bromophenol blue). Adrenal incubates were obtained as described above for electrophoresis, and concentrated. Non-denaturing tube polyacrylamide gels (7%), 80 × 6 mm i.d., were prepared as described by Davis [14]. The gels contained 10% glycerol and 2 nM [1,2-^{3}H]corticosterone and were run according to the steady state electrophoretic conditions of Ritzen *et al.* [15]. Dexamethasone was added in 1000 times excess to prevent non-specific binding. Sufficient protein to give equal [^{3}H]corticosterone binding with rat serum, adrenal incubate, and purified rat CBG, was loaded on the gel. Gels were run either at 2 or 22°C at a constant current of 2 mA/gel until the tracking dye (bromophenol blue) was 0.5 cm from the end of the gel. Gels were then frozen using dry-ice and sliced into 2 mm segments. The segments were left overnight in 3 ml scintillation fluid then counted in a scintillation counter.

Binding affinity

Scatchard analysis [16] was carried out to determine the apparent dissociation constant (K_d) of the specific binding proteins for corticosterone in both incubation medium and serum. The method used was that described in detail by Bassett [7]. Since the K_d for the binding of corticosteroids to CBG, determined by Scatchard analysis, varies with the dilution of plasma [17] the serum was diluted to 0.01% to obtain the same bound/free ratio as for the incubation medium.

Statistical analysis

Result are presented as mean ± SEM for the number of replicates indicated. Unless otherwise stated significance of differences between means was assessed using 1-way analysis of variance followed by Ryan's Q test for multiple comparison [18]. Statistical significance was set at $P < 0.05$.

RESULTS

Release of corticosterone and corticosterone-binding protein

The release of corticosterone from incubated adrenal slices after 30–60 min incubation is shown in Figs 2(A) and (B), respectively. At each incubation time ACTH(1–24) significantly increased the release of corticosterone in a dose dependent manner, in both the unpretreated and pretreated animals (1-way analysis of variance; 30 min unpretreated $F = 15.14$. (2,9), $P = 0.001$; pretreated $F = 18.45$ (2,9), $P = 0.001$; 60 min unpretreated $F = 28.72$ (2,9), $P < 0.001$; pretreated $F = 48.18$, (2,9), $P < 0.001$). Pretreatment with betamethasone as a whole significantly reduced the level of corticosterone release when compared to the unpretreated animals as a group (2-way analysis of variance: 30 min $F = 68.03$ (1,18) $P < 0.001$; 60 min $F = 53.11$, (1,18), $P < 0.001$. The corticosterone released after 60 min incubation was significantly greater than after 30 min incubation in all groups (unpaired t-test; $P < 0.05$ in all cases). Removal of calcium from the

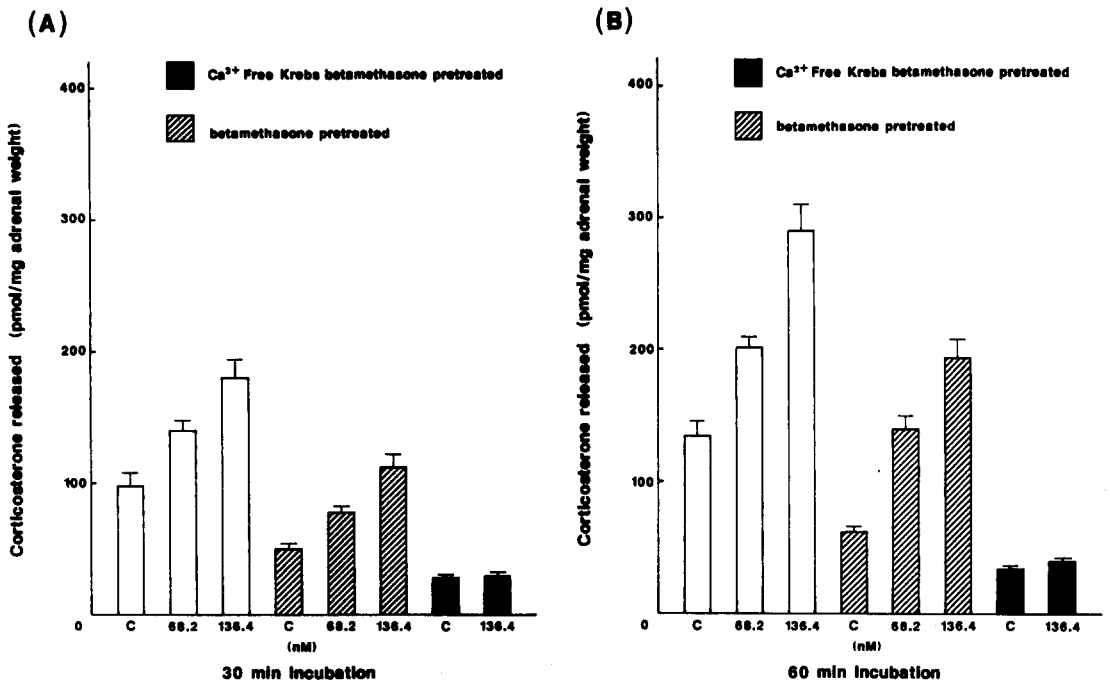

Fig. 2. Corticosterone released into medium containing adrenal slices after (A) 30 min and (B) 60 min incubation. Adrenal slices were either unstimulated control (C) or stimulated with 68.2 or 136.4 nM ACTH(1–24). Adrenals from animals not pretreated with betamethasone are represented by (□), animals pretreated with betamethasone by (■) and animals pretreated with betamethasone and calcium removed from the incubation medium by (■). Each column represents the mean of 4 incubations, horizontal bars designate ± SEM.

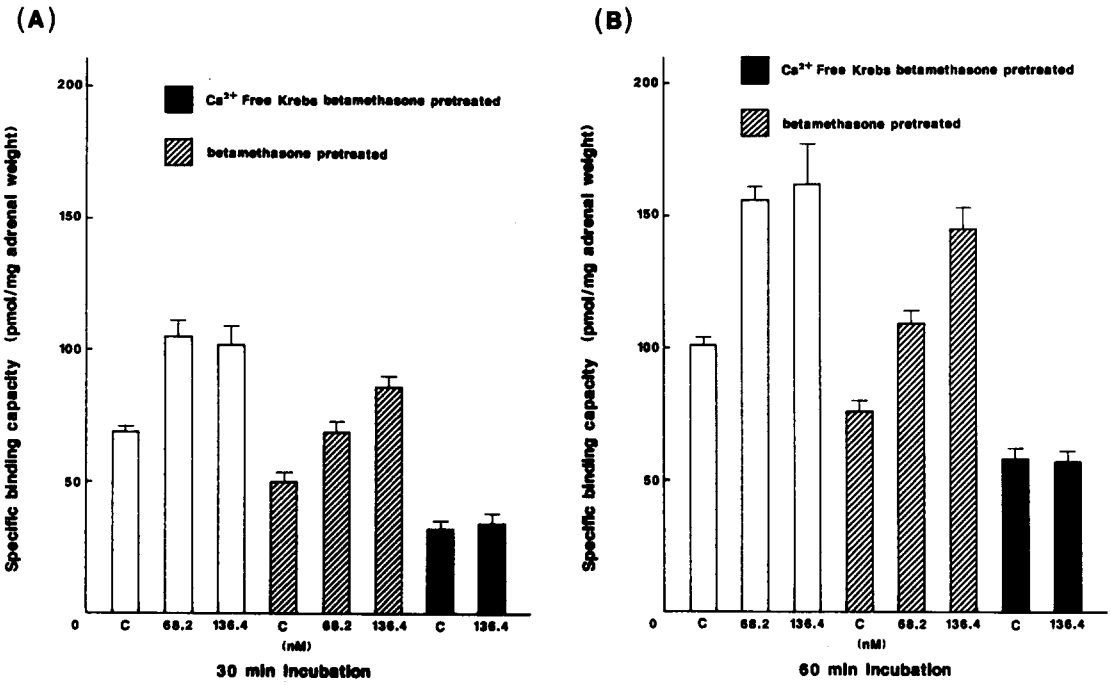

Fig. 3. Specific corticosterone binding capacity in medium containing adrenal slices after (A) 30 min and (B) 60 min incubation. Adrenal slices were either unstimulated controls (C) or stimulated with 68.2 or 136.4 nM ACTH(1–24). Adrenals from animals not pretreated with betamethasone are represented by (□), animals pretreated with betamethasone by (■) and animals pretreated with betamethasone and calcium removed from the incubation medium by (■). Each column represents the mean of 4 incubations, horizontal bars designate ± SEM.

incubation medium completely abolished the ACTH(1–24) induced release of steroid.

The increase in specific binding capacity from incubated adrenal slices after 30 and 60 min incubation is shown in Figs 3(A) and (B), respectively. At each incubation time ACTH(1–24) significantly increased the binding capacity in a dose dependent manner in the betamethasone pretreated animals (1-way analysis of variance: 30 min $F = 23.99$, (2,9), $P < 0.001$; 60 min $F = 40.40$, (2,9), $P < 0.001$. With the unpretreated animals there was no significant difference in the binding capacity following stimulation with either 68.2 or 136.4 nM ACTH(1–24), although both doses resulted in a significant increase in binding capacity compared to the unstimulated control group. As was the case for corticosterone release, pretreatment with betamethasone, handled as a single group, significantly reduced the binding capacity when compared to the unpretreated animals as a group (2-way analysis of variance: 30 min $F = 44.30$, (1,18), $P < 0.001$; 60 min $F = 25.89$, (1,18), $P < 0.001$). The binding capacity after 60 min incubation was significantly greater than after 30 min incubation (1-way analysis of variance; 30 min, $F = 19.04$, (1,22), $P = 0.001$; 60 min, $F = 14.15$, (1,22), $P = 0.001$). Removal of calcium from the incubation medium abolished the ACTH(1–24) induced release of steroid.

Specificity of binding

The ability of various steroids to compete for [^{3}H]corticosterone binding sites in rat serum and incubation medium is shown in Figs 4(A) and (B), respectively. Both serum and incubation medium gave very similar profiles. Corticosterone was the most potent competitor for [^{3}H]corticosterone binding sites. These profiles are in agreement with the affinity profile of rat plasma CBG reported by Feldman *et al.* [19] and the efficiency of competition by the various steroids tested is similar to the relative binding activity of each steroid for binding CBG [20].

Electrophoresis and Western blot analysis

The result of a Western blot of SDS–PAGE of both rat serum and adrenal incubate using anti-rat CBG as the primary anti-serum is shown in Fig. 5. Purified rat CBG gave a doublet band, the more intense band having

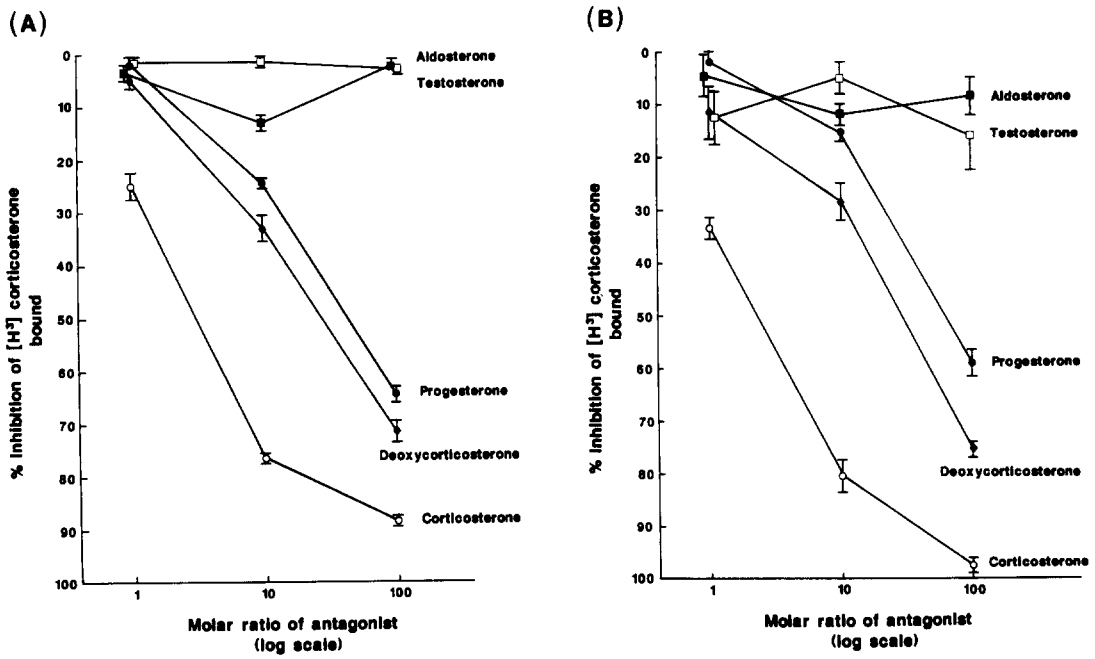

Fig. 4. Competition of steroids for [^{3}H]corticosterone binding sites in (A) diluted rat serum (0.1%) or (B) adrenal incubation medium. Serum or medium was stripped of endogenous steroids using DCC, then incubated with 1.2 nmol/l [^{3}H]corticosterone together with increasing concentrations of competing unlabelled steroid at 37°C for 30 min, then at 4°C for 15 min. Bound and free steroids were separated by DCC. Non-specific binding and binding to albumin or glucocorticoid receptor protein was blocked using 1000 times molar concentration of dexamethasone. Binding in the absence of competitor was taken as 0% inhibition. Each point represents the mean of 3 determinations. Horizontal bars designate ± SEM. Competing steroids were aldosterone (■), testosterone (□), progesterone (●), deoxycorticosterone (◆) and corticosterone (○).

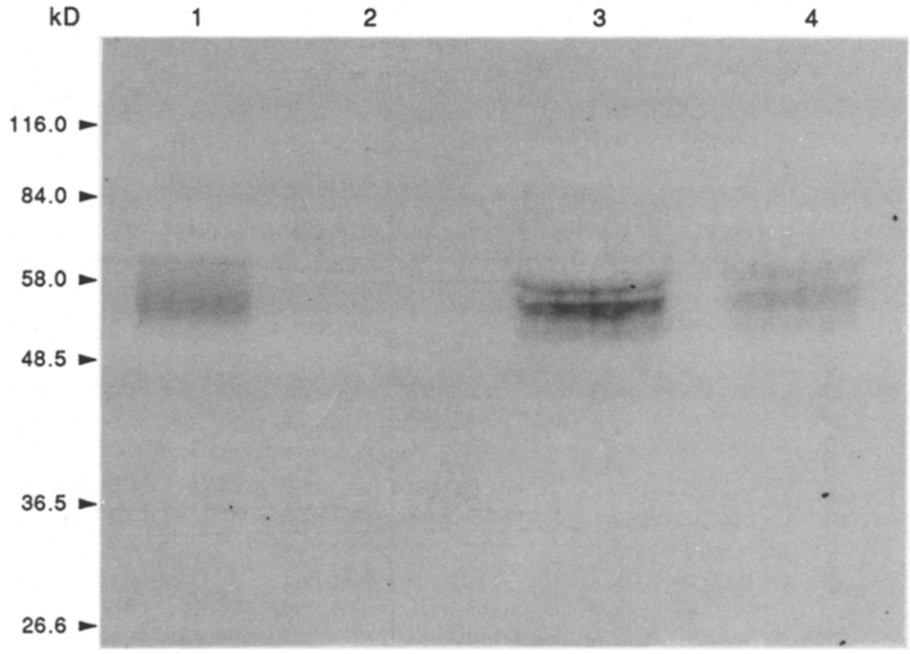

Fig. 5. Western blot of SDS–PAGE separation of rat serum and adrenal incubate. Samples were subjected to reducing SDS–PAGE (10%) and blotted onto nitrocellulose as described in Methods. The primary antibody was mouse anti-rat CBG and the secondary antibody was goat anti-mouse IgG linked to alkaline phosphotase. Track 1 is purified rat CBG; track 2 is adrenal incubate (2.5 μl: corticosterone binding capacity equivalent to track 3); track 3 is rat serum (2 μl) and track 4 is rat serum (1 μl).

a M_W of approx. 53,000 and the lighter band a M_W of 57,000. Kato *et al.* [21] also found that purified rat CBG ran as a doublet band in 7.5% SDS–PAGE gels, the strongest band was with a M_W of approx. 50,500. With rat serum a doublet band was observed also with M_Ws of approx. 55,000 and 57,000. With adrenal incubates no bands were observed using antiserum directed against rat CBG, even though the amount of incubate loaded had a greater corticosterone binding capacity than the serum.

Disc electrophoresis

The binding of [^{3}H]corticosterone to rat serum, purified rat CBG, and concentrated adrenal incubate at 2 and 22°C is shown in Fig. 6. Steady state electrophoresis run at 2°C gave only 1 peak for all 3 samples with approximately the same R_f value, indicating that the binding proteins present possessed similar ratios of net charge/surface area under the conditions of the electrophoresis [22]. Proteins with similar R_f values may have appreciably different M_Ws [21]. Gels run at 22°C showed the same single binding peak for serum and purified CBG. In the case of the adrenal incubate, however, at 22°C the binding of [^{3}H]corticosterone was no longer apparent.

Scatchard analysis

A Scatchard plot of [^{3}H]corticosterone binding to specific binding proteins in the incubation medium was linear over the range of steroid concentration examined (Fig. 7), indicating a single binding protein. The K_d values (mean $\pm$ SEM; $n = 5$) of the specific binding proteins from incubation medium and 0.01% rat serum were 3.66 ± 0.37 and 2.09 ± 0.16 nmol/l, respectively. An unpaired "t" test showed a significant difference ($P < 0.001$) between the 2 means, the diluted serum giving the lower K_d therefore the greater affinity.

DISCUSSION

As far back as 1974 Rubin *et al.* [4] concluded that the release of corticosteroid was accompanied by protein and occurred by a process of exocytosis. The current study supports the conclusion of steroid release being accompanied by a binding protein. The release of both steroid and binding protein was both dose and time dependent, and could be abolished by the removal of calcium from the incubation medium. In control animals the increased binding capacity could account for 100% of the steroid released, but this percentage progressively decreased as the level and duration of stimulation

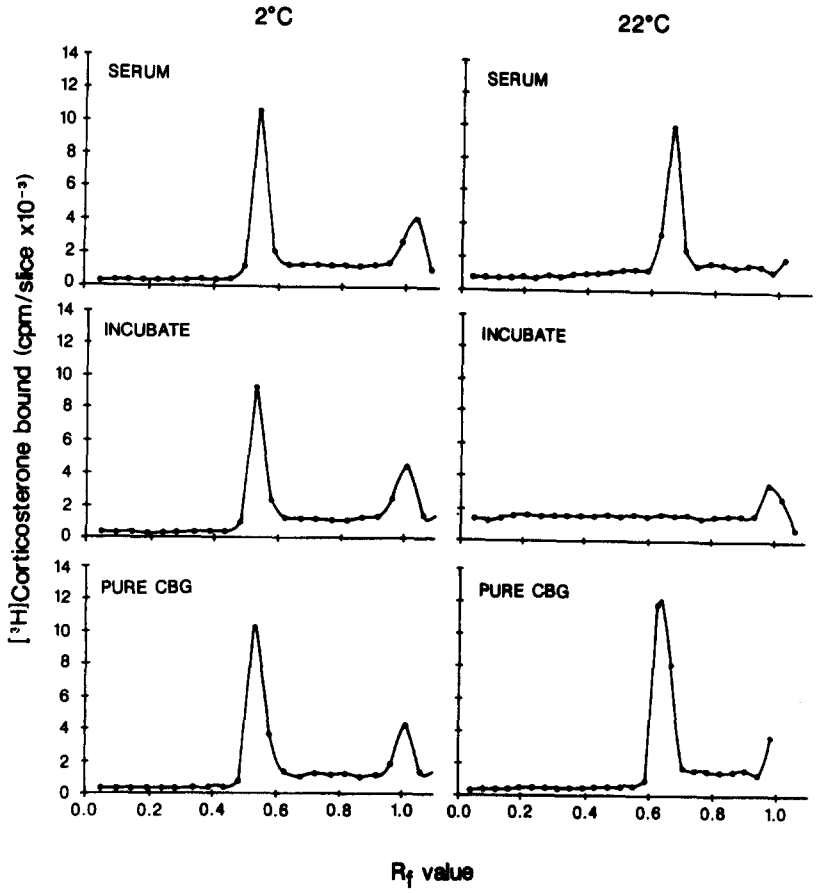

Fig. 6. Binding of [^{3}H]corticosterone to rat serum, purified CBG and adrenal incubate at 2 and 22°C. Non-denaturing tube polyacrylamide gels (7%) containing 2 nM [^{3}H]corticosterone were run according to the steady state conditions of Ritzen *et al.* [15]. After electrophoresis, the gels were cut into 2 mm slices and the radioactivity of each slice counted. Each point represents the activity of each slice starting from the cathode end of the gel. This experiment was repeated 3 times using different rat sera and incubation media. The small peak at front (anode end) originates from an association of corticosterone with the dye band (bromophenol blue) [32].

increased, being only 75% with 136.4 nM ACTH(1–24) after 60 min incubation.

Betamethasone pretreatment, which abolishes the endogenous release of ACTH [8] reduced both the steroid and binding protein release from the incubated adrenal slices. Such a finding would suggest that adrenals from the non-pretreated animals were in fact already stimulated by endogenous ACTH released during the short period of handling prior to killing. The release of steroid and protein from adrenals of non-pretreated animals being the result of stimulation by both endogenous and exogenous ACTH.

In the untreated animals, while a dose dependent release of corticosterone was observed, there appeared to be a limit to the increase in binding capacity. It has been proposed that the release of corticosterone on stimulation of the adrenal gland by ACTH(1–24) occurs in two phases; the initial phase is mainly release of corticosterone from a finite storage form within the gland, the second phase resulting from the release of newly synthesized hormone [23]. In the incubated adrenal slices the increase in corticosterone concentration at 30 and 60 min would be a combination of both sources since the stimulation of *de-novo* synthesis begins within 5 min and is at its maximum rate within 30 min [24]. While ACTH will stimulate both the synthesis and release of the glucocorticoids [25], stimulation of the synthesis of the binding protein may be impaired in the *in vitro* preparation. Whereas the increase in corticosterone represented the combination of stored and newly synthesized steroid, the increase in binding protein may represent mainly release from the limited storage form. Such a postulate is in agreement with the findings of Pritchett *et al.* [5] and Bassett [7]. Pritchett *et al.* [5] found that the corticosteroidogenic response to ACTH

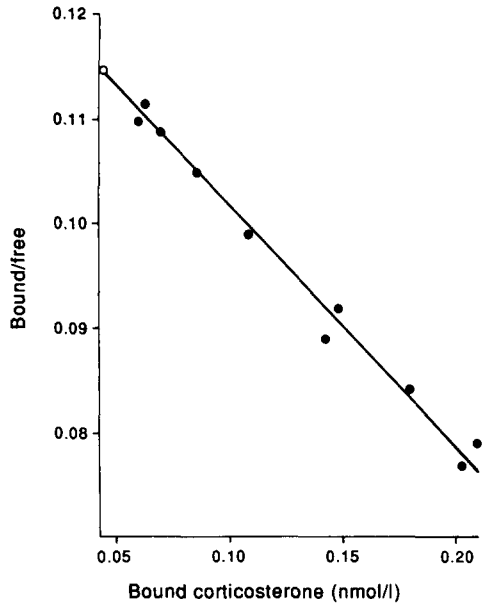

Fig. 7. Scatchard plot of [^{3}H]corticosterone binding to proteins from incubated adrenal slices in the presence of dexamethasone (0.9 nmol/l). Median was stripped of endogenous steroids using DCC. Stripped medium was incubated with the labelled steroid for 30 min at 37°C and then for 15 min at 4°C. Unbound corticosterone was removed using DCC at 4°C. Linear regression analysis gave a regression coefficient at 0.995 and an apparent K_d (the negative reciprocal of the slope) of 3.97 nmol/l. Circled points indicate duplicate points.

in incubated adrenal glands remained intact during ensuing incubations, but that the enhanced corticosterone binding capacity was only seen following the initial incubation period. Bassett [7], studying the enhanced corticosterone binding capacity in plasma after stimulation by stress or ACTH administration, concluded that while ACTH may be associated with the release of both steroid and binding protein, it did not appear to be responsible for stimulating the synthesis of the binding protein.

The existence of a mineralocorticoid binding protein of adrenal origin has been proposed previously by Raven *et al.* [26]. In the current study, however, the specificity of steroid binding to the adrenal incubate was almost identical to that of CBG in rat serum, indicating that the binding protein was not specifically associated with other steroid hormones released from the adrenal cortex. The possibility that the binding capacity in the incubate is an artifact, representing serum CBG which transiently becomes dissociated from adrenocortical tissue, was not supported by the dose and calcium dependent nature of its release. It was also not supported by the finding that the monoclonal antibody against rat CBG failed to recognize the adrenal protein, while recognizing purified rat CBG and CBG in rat serum.

The adrenal binding protein would appear to be more labile than CBG, particularly when not bound to corticosterone. Stripping of endogenous steroid with DCC was found to dramatically reduce the binding capacity of the incubate while not affecting the binding capacity of serum. While disc gel electrophoresis showed that the adrenal binding protein and CBG ran with the same R_f value, the adrenal protein was more temperature sensitive. Bassett [7] also concluded that the proposed adrenal protein was very labile since enhanced binding in blood disappeared within 10 min. The decline in binding capacity may represent a functional inactivation once the steroid is stripped from its binding site. In the guinea-pig and rabbit, on removal of bound steroid rapid self association of CBG to form polymers is reported to lead to inactivation of the binding protein [27]. Depolymerization and reactivation of the binding protein could only be obtained by incubation with a relatively large quantity of corticosteroid [27]. In the present study in order to get maximum binding capacity it was necessary to incubate the adrenal medium with relatively large quantities of corticosterone (36.3 nmol; 8 times the endogenous steroid level), suggesting that a similar polymerization may be occurring with the adrenal protein when stripped of the corticosteroid.

Comparing the K_d from animals with functional and non-functional adrenals, Bassett [7] concluded that any adrenal binding protein must have a lower affinity for corticosterone than CBG. This conclusion is supported by the current study where Scatchard analysis of corticosterone binding indicated a significantly greater K_d for the incubation medium than for diluted serum.

Several advantages for such an adrenal protein are apparent: (1) solubility of the steroid hormone within the secretory vesicle and its containment within the vesicle; (2) economy of hormone action since once released the protein bound corticosterone would be protected against movement down concentration gradients into non-target cells in the region due to the lipid nature of the hormone and (3) binding capacity would match the level of steroid secretion. This is not the case with CBG where its release from the liver is reported to be either not affected by glucocorticoid or ACTH levels [28] or to be inhibited by the glucocorticoids [19]. It

is proposed that once released the adrenal protein serves to carry the steroid hormone from the intercellular space to the plasma, where due to the lower affinity of the adrenal protein the steroid may be transferred to CBG not already bound to corticosterone.

The present results do not indicate whether the adrenal protein arises from *de-novo* synthesis within the adrenal cells, or by sequestration and subsequent modification of circulating CBG. CBG-like molecules have been identified within cells of a number of target tissues and it has been suggested that such intracellular CBG can arise from the circulation [29]. Singer *et al.* [30] presented data from the rat which were consistent with an internalization mechanism involving binding of CBG to the membrane, followed by subsequent metabolism of the protein to internalizable species. Such a sequence of events would explain the reported dissimilarities in the properties of intracellular and plasma CBG. Rat muscle CBG has been shown to exhibit a decreased affinity for cortisol [31]. Whether this mechanism applies to the adrenal protein is currently under investigation.

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