0013-7227/88/1233-1642$02.00/0
Endocrinology

Vol. 123, No. 3
Printed in U.S.A.

[^{3}H]Dexamethasone Binding to Plasma Membrane-Enriched Fractions from Liver of Nonadrenalectomized Rats*

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ABSTRACT. Using liver from nonadrenalectomized adult male rats, binding sites for [^{3}H]dexamethasone in particulate fractions are demonstrated. The binding is thermolabile, saturable, and specific for glucocorticoid. The apparent dissociation constant ($K_d$app) for [^{3}H]dexamethasone (0.48 ± 0.084 μM) is 60-fold greater than that for cytosolic receptor (7.9 ± 1.5 nM). The $K_d$app for [^{3}H]cortisol in particulate fractions is 2.5-fold lower than for [^{3}H]dexamethasone ($K_d$app = 0.18 μM). The binding capacities for particulate and cytosolic glucocorticoid-binding sites also differ significantly, with particulate sites at least 9.1-fold more concentrated than cytosolic sites in liver tissue. Particulate sites are determined in Percoll density gradients to have a density of 1.039 g/cc. Saturable [^{3}H]dexamethasone radioactivity coelutes from these gradients with the plasma membrane marker enzyme 5′-nucleotidase. Adrenalectomy causes the complete loss of particulate binding sites by 6 days postadrenalectomy; however, these sites can be regenerated to two thirds of the nonadrenalectomy level by 20–30 days postadrenalectomy (*Endocrinology* **123:** 1642–1651, 1988)

A REVIEW of the literature on the cellular distribution of steroid hormone receptors supports the presence of steroid-binding sites associated with several subcellular fractions. For all classes of steroid hormone, evidence exists for binding to target cell plasma membranes, mitochondria and/or lysosomes, and microsomal fractions. [For an extensive literature review, see Szego and Pietras (1).] However, the lack of documentation for these binding sites in all hormone-sensitive tissues has failed to engender support for their significance to hormonal actions. Furthermore, the lack of consistency in their reported properties has not supported their separate identity as binding sites. For example, the binding of [^{3}H]corticosterone to rat pituitary plasma membrane was compared by Koch *et al.* (2) with binding to transcortin, and the low percentage of [^{3}H]estradiol binding to purified uterine plasma membrane from immature female rats was concluded by Muller *et al.* (3) to result from contamination by cytosolic receptor. Recently, Gametchu (4) demonstrated a glucocorticoid-like receptor molecule on the membrane surface of S49 mouse lymphoma cells, using immunofluorescent labeling with a monoclonal antibody made to the soluble cytosolic receptor.

Received November 13, 1987.
* This work was supported in part by Grant AM-37821-01 from the NIH, a DuPont College Science Grant in Chemistry, and the Middlebury College Faculty Professional Development Fund.
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In the present studies evidence is presented for [^{3}H]dexamethasone-binding sites in particulate preparations from rat liver. These binding sites appear to be associated with the plasma membrane. Furthermore, the progressive decrease and eventual loss of such binding sites in liver from adrenalectomized rats are demonstrated. The universal use of adrenalectomy is thought to be the major reason that these sites have been overlooked. The rat liver is well known for its physiological responsiveness to glucocorticoids (5–8). It is well characterized for subcellular fractionation by centrifugation (9), thereby providing a reference for the estimation of subcellular localization of specific binding sites. Furthermore, the cytosolic glucocorticoid receptor from rat liver has been well characterized (10–12) and provided a reference for comparison of particulate binding sites with those characteristic of soluble receptor species.

Materials and Methods

Animals

Intact (nonadrenalectomized) and adrenalectomized adult male Long-Evans hooded rats were obtained from Charles River Laboratories (St. Constant, Quebec, Canada). Rats were maintained on Purina rat chow (Ralston-Purina, St. Louis, MO). Adrenalectomized rats were provided water supplemented with 0.9% NaCl.

Chemicals

[6,7-^{3}H]Dexamethasone (39.4–45.8 Ci/mmol) and [1,2-^{3}H] hydrocortisone (cortisol; 51.0 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Calf thymus DNA, Concanavalin-A-agarose (Con-A-agarose) type V-A (10 mg protein/ml packed gel), dexamethasone, cortisol, D-mannose, α-methyl-D-mannose, β-mercaptoethanol, and 3-(*N*-morpholino)-propanesulfonic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium molybdate, potassium chloride, and toluene were obtained from Fisher Scientific (Pittsburgh, PA). Econo-Column chromatography columns (id, 0.7 mm) were obtained from Bio-Rad Laboratories (Richmond, CA). All other chemicals were reagent grade and obtained from Sigma Chemical Co., Fisher Scientific or Aldrich Chemical Co. (Milwaukee, WI).

Tissue preparation

Rats were killed by decapitation. Liver was perfused and rinsed in MMM buffer (10 mM 3-(*N*-morpholino)-propanesulfonic acid, 12 mM β-mercaptoethanol, and 10 mM sodium molybdate, pH 6.5; 4 C) or MMMS (MMM buffer made 0.25 M in sucrose). Tissue was homogenized with a Dounce homogenizer (Kontes Co., Vineland, MI) in 3 vol (1:3, wt/vol) MMM buffer. This homogenization and all subsequent procedures were carried out at 4 C unless otherwise specified. The resulting suspension was called crude homogenate. Crude homogenate was centrifuged at 600 × *g* for 15 min to remove nuclei and large cellular particles. The resulting suspension was called homogenate. Homogenate was further centrifuged at 117,000 × *g* for 1 h, to produce supernatant (soluble or cytosolic fraction) and pellet (particulate fraction). For differential centrifugation, MMMS buffer was employed, and the following subcellular fractionation was performed to remove subcellular fractions from homogenate (9): 25,000 × *g* for 10 min (lysosomes, mitochondria, and peroxisomes), 34,000 × *g* for 30 min (heavy microsomes), and 117,000 × *g* for 60 min (light microsomes).

Determinations of steroid binding

Steroid incubations were performed with [^{3}H]dexamethasone or [^{3}H]cortisol and evaporated to dryness before the addition of tissue preparations. To determine nonsaturable binding, parallel incubations were performed with an excess of unlabeled dexamethasone. Bound [^{3}H]dexamethasone was determined by dextran-coated charcoal (DCC) assay (13) or GF/B filtration assay (14). For competitive binding studies, an excess of unlabeled 17β-estradiol, progesterone, cortisol, testosterone, dexamethasone, or androstanediol was added with [^{3}H] cortisol.

Protein and enzyme assays

Protein was assayed by the Bio-Rad Standard Protein Assay using BSA as a standard. 5′-Nucleotidase was assayed as described by Touster *et al.* (15), except that the assay was allowed to proceed for 2.5 h before termination with the addition of 30% trichloroacetic acid. Glucose-6-phosphatase was determined by the method of Nordlie and Arion (16) with a similar modification of the incubation time to 2.5 h.

Kinetic analysis

For Lineweaver-Burk and saturation analyses, [^{3}H]dexamethasone was incubated at concentrations ranging from 2.63 nM to 0.10 μM for supernatant incubations and 9.0 nM to 1.7 μM for pellet incubations. [^{3}H]Cortisol was incubated at concentrations ranging from 75.5 nM to 1.38 μM for pellet incubations. Each point represents the mean of six individual determinations. Data were fitted by means of a linear least squares computation curve-fitting program, and the fit was determined by r^2; only data where $r^2 > 95\%$ was used. Errors are expressed as SDs.

Con-A-agarose chromatography

Columns contained 3.6 ml Con-A-agarose (type V-A) packed into glass Econo-columns (id, 7 mm). Columns were prepared by washing with at least 3 bed vol 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM $MnCl_2$. [^{3}H]Dexamethasone incubate was treated with DCC, and 0.100 ml was loaded onto the column, followed by 0.200 ml buffer. The column was allowed to equilibrate for 10 min. Approximately 8 ml MMM were then washed through the column and 50 5-drop fractions were collected. KM solution (0.5 M KCl and 0.3 M α-methyl mannose in MMM) was then applied, 10 min of equilibration were allowed, and a second set of 50 fractions was collected. Even-numbered fractions were collected, and 5 ml scintillation fluid were added [5% POPOP (1,4-bis-[2-(5-phenyloxazolyl)]benzene) and 0.5% PPO (2,5-diphenyloxazole) in toluene] to each tube before determination of radioactivity in a Packard 3330 liquid scintillation spectrometer (Packard, Downers Grove, IL).

Percoll density gradients

Self-generating 13.5-ml Percoll gradients were prepared at a starting density of 1.06 g/cc, made isoosmotic with sucrose (0.25 M) in 13.5-ml Ultra-Clear centrifuge tubes (Beckman, Palo Alto, CA), and centrifuged for 25 min at 100,000 × *g* in a type 65 rotor. Gradients were eluted from the bottom, and five-drop fractions were collected. Gradients were standardized using Density Marker Beads (Pharmacia, Piscataway, NJ) with densities of 1.035, 1.050, 1.070, and 1.083 g/cc in Percoll containing 0.25 M sucrose.

Results

To remove specific circulating steroid hormones, organectomy has been routinely performed before characterization studies for cytosolic and nuclear steroid hormone receptors. In the proposed studies, it was questioned whether the performance of adrenalectomy to remove endogenous glucocorticoid could cause changes in subcellular preparations and alter glucocorticoid binding. This consideration was of particular concern, as glucocorticoids are well known effectors of membrane structure (17, 18). A rapid simple method was sought

which would permit a qualitative comparison of specifically bound radiolabeled glucocorticoid in subcellular fractions from adrenalectomized and intact rat liver membranes. Immobilized lectin chromatography was considered, as it has been extensively used for the fractionation of cell populations. Although this technique has not yet been employed for separation of subcellular fractions, such an application has been suggested (19). Con-A-agarose was chosen as a membrane probe, as it provides an immobilized lectin surface capable of fractionating cellular species based on highly discriminate association with specific carbohydrate substituents of glycoproteins or glycolipid as well as resolution by size on agarose beads.

Homogenate, containing suspended cellular membranes from intact nonadrenalectomized rat liver, was prepared by disrupting liver tissue in a Dounce homogenizer to obtain crude homogenate, then centrifuging at 600 × *g* for 15 min to remove nuclei and large cell fragments. The homogenate was incubated for 2 h with [^{3}H]dexamethasone and chromatographed on 3.6-ml Con-A-agarose columns. The [^{3}H]dexamethasone-treated homogenate fractioned into five distinct peaks of saturable [^{3}H]dexamethasone binding on Con-A-agarose chromatography (Fig. 1, ■—■). Three distinct [^{3}H]dexamethasone binding peaks were eluted without retention to the Con-A-agarose. These peaks were designated peak P1 (fractions 12–14), peak CR (fractions 18–20), and peak V_t, corresponding to an elution at the total column volume (V_t) at fractions 28–30 (for peak designations, see description for Fig. 2 below). The separation of these nonabsorbing fractions can be attributed to low affinity interactions with Con-A and/or mol wt differences resolved by filtration through agarose gel. Two additional fractions are eluted with KM and are presumed to be bound to Con-A through carbohydrate-specific interaction. These populations represent broad overlapping peaks designated P2, eluting at fractions 66–70, and P3, eluting at fractions 74–78.

Homogenate from adrenalectomized rat liver was similarly incubated with [^{3}H]dexamethasone and chromatographed on Con-A-agarose. The elution profile of saturable [^{3}H]dexamethasone binding from 4-day postadrenalectomy rat liver exhibits a significantly increased presence of peak CR relative to P1 (Fig. 1, □– –□). Six days postadrenalectomy, peak P1 is present only as a small shoulder eluting before the predominant peak CR (△— —△). Peaks P2 and P3 are progressively decreased in postadrenalectomy animals. The elution profile on day 12 postadrenalectomy showed a singular large elution peak of CR, with all other peaks absent (▲— - —▲). The effect of adrenalectomy on rat liver [^{3}H]dexamethasone-binding fractions resolved by Con-A-agarose was concluded to be significant. The loss of heterogeneous

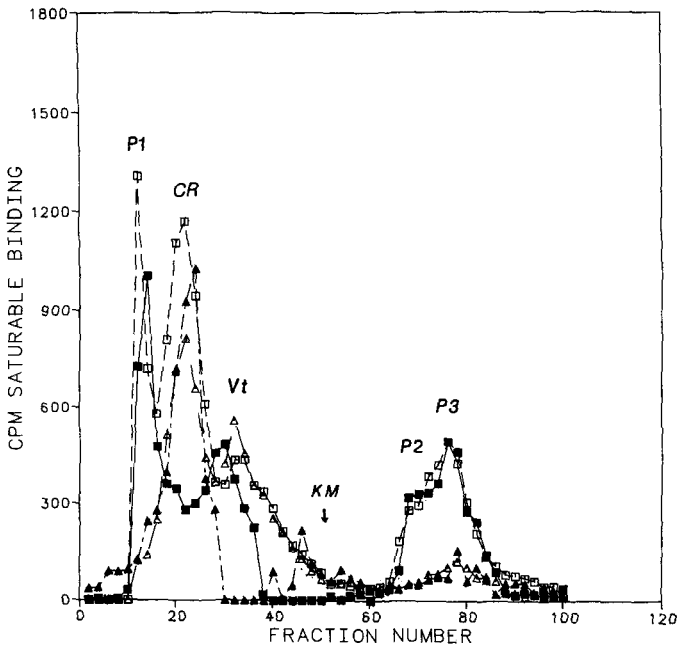

FIG. 1. Con-A-agarose chromatography of intact and adrenalectomized rat liver homogenate. Homogenate was prepared from an intact rat (■—■) or rats adrenalectomized 4 days (□——□), 6 days (△— —△), or 12 days (▲— - —▲) before experimentation. Homogenate was incubated for 2 h at 4 C with 20 nM [^{3}H]dexamethasone in the presence and absence of an approximately 1000-fold excess of unlabeled dexamethasone. After DCC treatment, a 0.100-ml aliquot was chromatographed on a 3.6-ml Con-A-agarose column. The column was eluted first with buffer (MMM), and 50 fractions were collected; then 0.300 ml KM applied and equilibrated, and 50 additional fractions were collected. Saturable counts per min were determined by subtracting nonsaturable from total binding, as described in *Materials and Methods*.

[^{3}H]dexamethasone-binding fractions after adrenalectomy suggested the presence of heretofore unrecognized cellular glucocorticoid-binding sites. These sites have been routinely overlooked due to the common practice of adrenalectomy before experimentation.

The approximate subcellular localization of these sites was evaluated next by differential centrifugation. Centrifugation of radiolabeled rat liver homogenate at 25,000 × *g*, 34,000 × *g*, and 117,000 × *g* showed that [^{3}H] dexamethasone binding was associated with several different subcellular fractions (Table 1). Significantly, under the conditions of incubation, over 70% of the total homogenate radioactivity of 21,000 dpm was associated with the particulate fractions. Only about 30% of the radioactivity was associated with the cytosolic fraction represented by the 117,000 × *g* soluble fraction (7,100 dpm).

Each centrifuged fraction was subjected to chromatography on Con-A-agarose, and the effect of centrifugation on Con-A-agarose-binding fractions was evaluated. The results are shown in Fig. 2 (A, supernatant; B, pellet). Peak CR was found only in the supernatant fractions

TABLE 1. Distribution of [^{3}H]dexamethasone binding in cellular fractions of rat liver

	[^{3}H]Dexamethasone binding	
	dpm	dpm/mg protein
Homogenate	21,100 (590)	14.8 (1.87)
25,000 × *g* supernatant	17,800 (560)	15.3 (0.017)
25,000 × *g* pellet	4,880 (37)	17.0 (0.002)
34,000 × *g* supernatant	11,400 (480)	11.4 (0.031)
34,000 × *g* pellet	7,000 (86)	24.0 (0.023)
117,000 × *g* supernatant	7,100 (47)	8.5 (0.06)
117,000 × *g* pellet	4,600 (410)	20.9 (1.87)

Rat liver homogenate was prepared in MMMS at 4 C and incubated with 20 nm [^{3}H]dexamethasone at 4 C for 2 h. The homogenate was subjected to differential centrifugation at 25,000 × *g* for 10 min, 34,000 × *g* for 30 min, and 117,000 × *g* for 1 h. Triplicate samples (0.050 ml) were assayed for radioactivity and protein content, and data are represented as disintegrations per min of [^{3}H]dexamethasone binding and as specific [^{3}H]dexamethasone binding (disintegrations per min/mg protein). SDs are shown in parentheses.

and in none of the pellet fractions, and was the only major peak observed in the 117,000 × *g* supernatant elution profile (Fig. 2A, ▲– –▲). This peak was termed CR with reference to the classic cytosolic receptor that is prepared by centrifugation under similar conditions (10, 12). Peaks P1, P2, and P3 were present in all fractions, except for the 117,000 × *g* supernatant. These three peaks were distinguished as particulate fractions (P) and are present in both the heavy (25,000 × *g* and 34,000 × *g* pellets) and light (117,000 × *g* pellet) microsomal fractions (Fig. 2B, ▲– –▲). Peaks P2 and P3 represent particulate fractions that are associated with Con-A, as chromatography through the agarose alone produced no evidence of these peaks. The peak at V_t was present in both supernatant and particulate fractions.

Binding of [^{3}H]dexamethasone to specific subcellular fractions was further supported by density gradient centrifugation studies using Percoll in 0.25 M sucrose. Homogenate, 117,000 × *g* pellet, and 117,000 × *g* supernatant (0.200 ml) were applied to separate gradients and centrifuged for 25 min at 100,000 × *g*. Fractionation of homogenate demonstrated two [^{3}H]dexamethasone-binding fractions (Fig. 3A). The major fraction (~70%) corresponded to a density of 1.039 g/cc. A second population (~30%) eluted at the top of the gradient and could not be further resolved on gradients of lowered starting density. Supernatant from 117,000 × *g* centrifugation of [^{3}H]dexamethasone-labeled homogenate similarly showed a sedimentation profile with a single peak corresponding to the low density homogenate peak. The 117,000 × *g* pellet fraction showed a single peak of 1.039 g/cc, corresponding to the major peak obtained in the crude homogenate gradient sedimentation. In a separate experiment, this single peak eluted exactly with the peak of 5′-nucleotidase (plasma membrane) activity at a density of 1.039 g/cc (Fig. 3B). Glucose-6-phosphatase (endoplasmic reticulum) activity eluted in a similar sharp band, but was consistently found four fractions earlier at a density of 1.045 g/cc in Percoll. Similarly, citrate

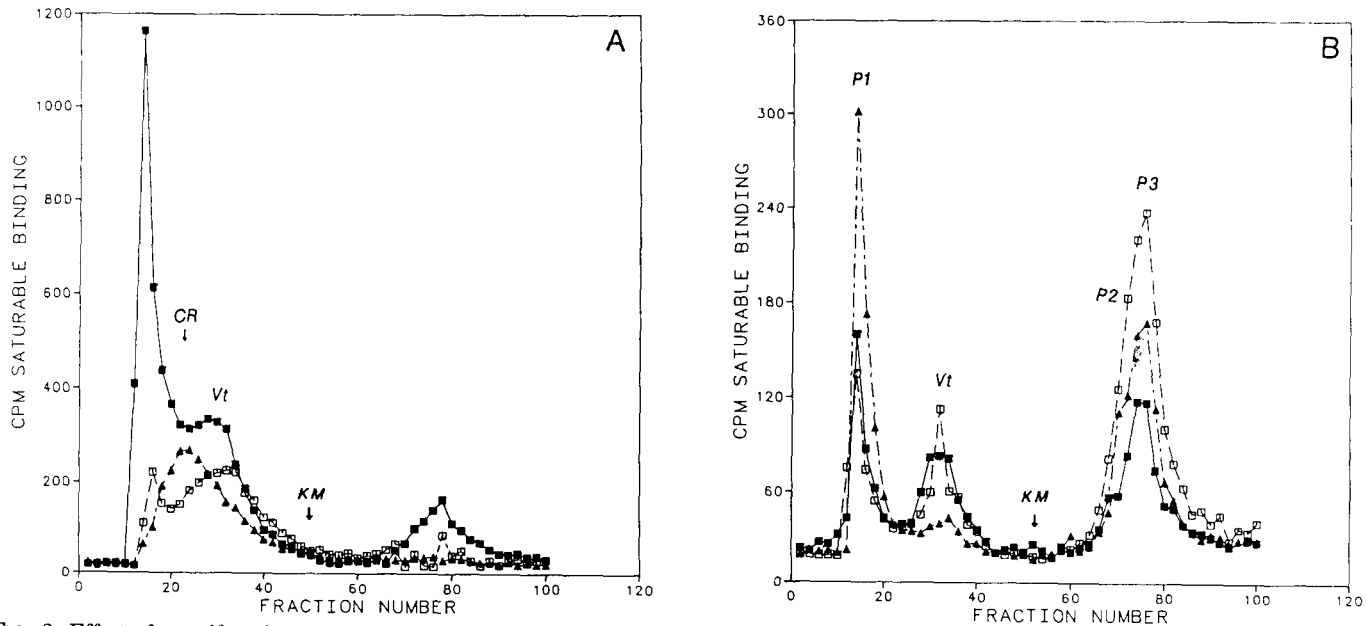

FIG. 2. Effect of centrifugation of rat liver homogenate on Con-A-agarose chromatography profile. Rat liver homogenate was incubated with 20 nM [^{3}H]dexamethasone for 2 h and then centrifuged at 25,000 × *g* for 10 min, 34,000 × *g* for 30 min, and 117,000 × *g* for 1 h (9). Pellets were removed after each centrifugation and resuspended to the original volume. Each fraction was then chromatographed on Con-A-agarose, as described in Fig. 1. A, Supernatant; B, pellet for 25,000 × *g* (■—■), 34,000 × *g* (□— —□) and 117,000 × *g* (▲— – —▲) centrifugation.

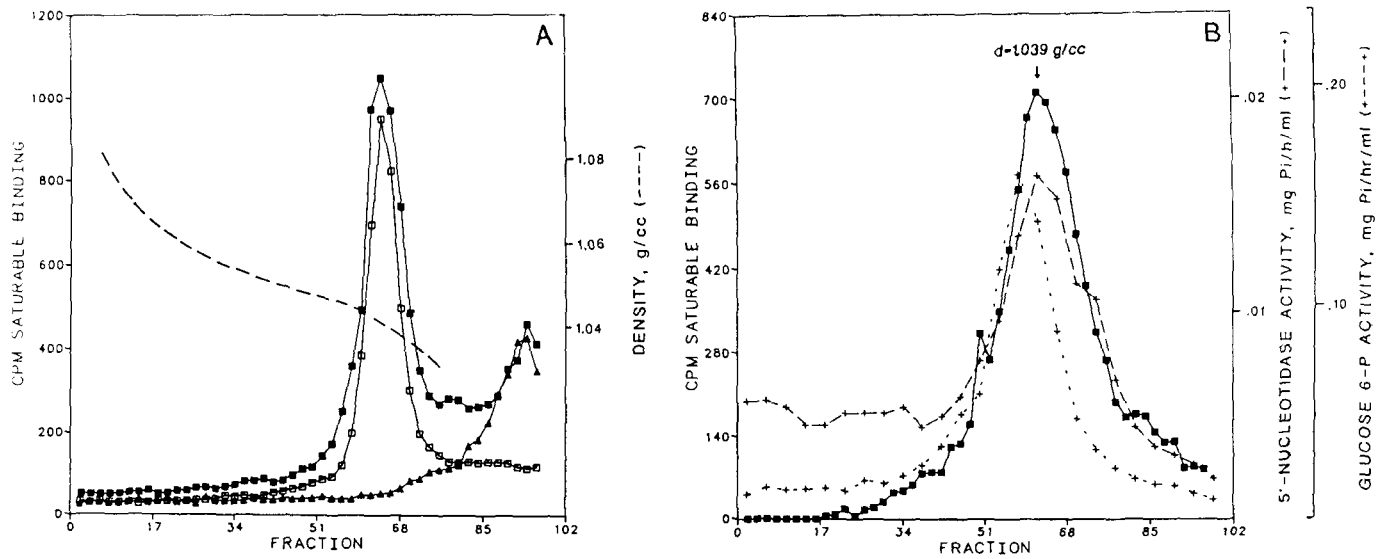

FIG. 3. A, Percoll density gradient centrifugation of rat liver homogenate, 117,000 × *g* supernatant, and 117,000 × *g* pellet. Homogenate was incubated with 20 nM [^{3}H]dexamethasone for 2 h and centrifuged at 117,000 × *g* for 1 h. Supernatant was removed, and pellet was resuspended to the original volume. Aliquots were treated with DCC, and 0.200 ml was applied to Percoll density gradients (13.5 ml) prepared at a starting density of 1.06 g/cc, made 0.25 M in sucrose. Gradients were centrifuged at 100,000 × *g* for 25 min and evaluated for radioactivity. Gradients were standardized using Density Marker Beads. All experiments were performed in parallel with excess unlabeled dexamethasone, and data are represented as saturable counts per min. ■—■, Homogenate; □—□, 117,000 × *g* pellet; ▲—▲, 117,000 × *g* supernatant. B, 5′-Nucleotidase and glucose-6-phosphatase activity in Percoll density gradient fractions of the 117,000 × *g* pellet. The pellet from the 117,000 × *g* centrifugation of [^{3}H]dexamethasone-labelled homogenate was centrifuged on Percoll density gradients as described in A, and fractions were evaluated for 5′-nucleotidase activity (+— —+), glucose-6-phosphatase activity (+– –+), and radioactivity (■—■).

synthase (mitochondrial) and UDP-galactosyl transferase (Golgi) activities were identified in fractions representing higher densities which did not correspond to the major [^{3}H]dexamethasone-binding peak.

Under the conditions of incubation, it was clear that the majority (~70%) of the saturable [^{3}H]dexamethasone binding was associated with particulate fractions enriched for the plasma membrane marker enzyme 5′-nucleotidase. It was considered that the approximately 30% saturable [^{3}H]dexamethasone binding observed in the 117,000 × *g* supernatant fraction might result from removal of steroid-binding macromolecules from the membrane during homogenization procedures. However, as shown in Table 2, 91% of the 5′-nucleotidase activity was associated with the 117,000 × *g* pellet fraction (25.8 U), and less than 9% was present in the supernatant fraction (2.46 U). This suggests that the conditions of homogenization and incubation preserve the integrity of the plasma membrane and, by analogy, glucocorticoid-binding sites associated with this membrane. Furthermore, the enrichment of 5′-nucleotidase in the 117,000 × *g* pellet (2.9) parallels the increased specific radioactivity in the pellet fraction (3.2), assuming distinct supernatant and particulate steroid-binding forms. That is, if one assumes that the distribution of [^{3}H]dexamethasone-binding sites into 117,000 × *g* pellet (67%) and supernatant (33%) fractions is a true reflection of different glucocorticoid binders, then 67% of the homogenate [^{3}H]dexamethasone binding (338 dpm/mg) is enriched 3.2-fold in the pellet fraction (1,070 dpm/mg).

TABLE 2. Distribution of [^{3}H]dexamethasone binding and 5′-nucleotidase activity in 117,000 × *g* subfractions from rat liver

	[^{3}H]Dexamethasone binding		5′-Nucleotidase activity	
	dpm	dpm/mg protein	U[a]	Specific U[b]
Homogenate	21,870 (468)	505 (19.7)	28.3 (0.108)	0.654 (0.0141)
117,000 × *g* supernatant	7,300 (134)	234 (33.3)	2.46 (0.136)	0.0788 (0.0141)
117,000 × *g* pellet	14,500 (497)	1,070 (59.1)	25.8 (0.467)	1.91 (0.0745)

Rat liver homogenate was prepared in MMMS and incubated in the presence of 20 nM [^{3}H]dexamethasone at 4 C for 2 h. The homogenate was centrifuged at 117,000 × *g* for 1 h. Triplicate samples (0.050 ml) of each fraction were then assayed for radioactivity or 5′-nucleotidase activity, as described in *Materials and Methods*. SDs are shown in parentheses.

[a] One unit of enzyme is defined as milligrams of phosphate released per h/ml at 37 C.

[b] Units per mg protein.

It was concluded that [^{3}H]dexamethasone binds to plasma membrane-enriched fractions from nonadrenalectomized rat liver as well as to a soluble receptor form. These forms can be separated quantitatively by centrifugation at 117,000 × *g*. Under the conditions of incubation, the majority (~70%) of the [^{3}H]dexamethasone binding was associated with the particulate fraction and, more specifically, with a plasma membrane-enriched fraction. The remaining approximately 30% [^{3}H]dexamethasone binding was found in the 117,000 × *g* supernatant fraction and was unlikely to result from particulate binding sites disrupted during the homogenization and incubation conditions.

[^{3}H]Dexamethasone binding to the 117,000 × *g* particulate fraction was further compared to [^{3}H]dexamethasone binding obtained for the 117,000 × *g* supernatant. At 4 C, specific [^{3}H]dexamethasone binding increased over time up to 21 h (Fig. 4). At 20 and 30 C, maximal binding was reached more rapidly and was thermolabile for both the cytosolic and particulate fractions. Supernatant and pellet incubations at 4 C do not attain equilibrium conditions by 45–90 min, as reported in previous studies for the soluble cytosolic receptor from adrenalectomized animals (11, 12). However, the continual dissociation of receptor sites occupied by endogenous steroid would cause an increase in total available binding sites with time. Therefore, the conditions for equilibrium were evaluated in another manner.

Specific binding assays of cytosolic and membrane fractions were performed at 1.5, 11, 24, and 48 h, and the results are shown in Table 3. For the 117,000 × *g* particulate fraction, the number of binding sites (n) increased with time, while the apparent dissociation constant ($K_d$app) did not vary significantly. Since $K_d$app decreased only slightly with time, it was assumed that equilibrium with available sites was approximated by 1.5 h.

In contrast to particulate fraction, specific binding studies performed on 117,000 × *g* supernatant demonstrated little change in n, while $K_d$app increased 8-fold over the course of 48 h. Such a trend is characteristic of competitive inhibition by an inhibitor increasing in concentration. In fact, $K_d$app may be correlated with time (t) of incubation in a second order polynomial equation (r^2 = 100%):

$$K_d\text{app} = 6.96\ \text{nM} + (0.0297\ \text{nM/h})\,t + (0.0215\ \text{nM/h}^2)t^2 \quad \text{(I)}$$

$K_d$app may be related to K_d, K_i (dissociation constant for the receptor-inhibitor complex), and [I] (inhibitor concentration) as follows:

$$K_d\text{app} = K_d + K_d[I]/K_i \quad \text{(II)}$$

If Eq II is substituted into Eq 1 and the derivative of [I] taken with respect to time, the following rate expression is obtained for the formation of I:

$$d\,[I]/dt = (K_i/K_d)(0.0297\ \text{nM} + 0.0430\ \text{nM/h}\ t) \quad \text{(III)}$$

While this does not appear to represent a straight forward first or second order mechanism for the production of I, it could be explained by a two-step sequential

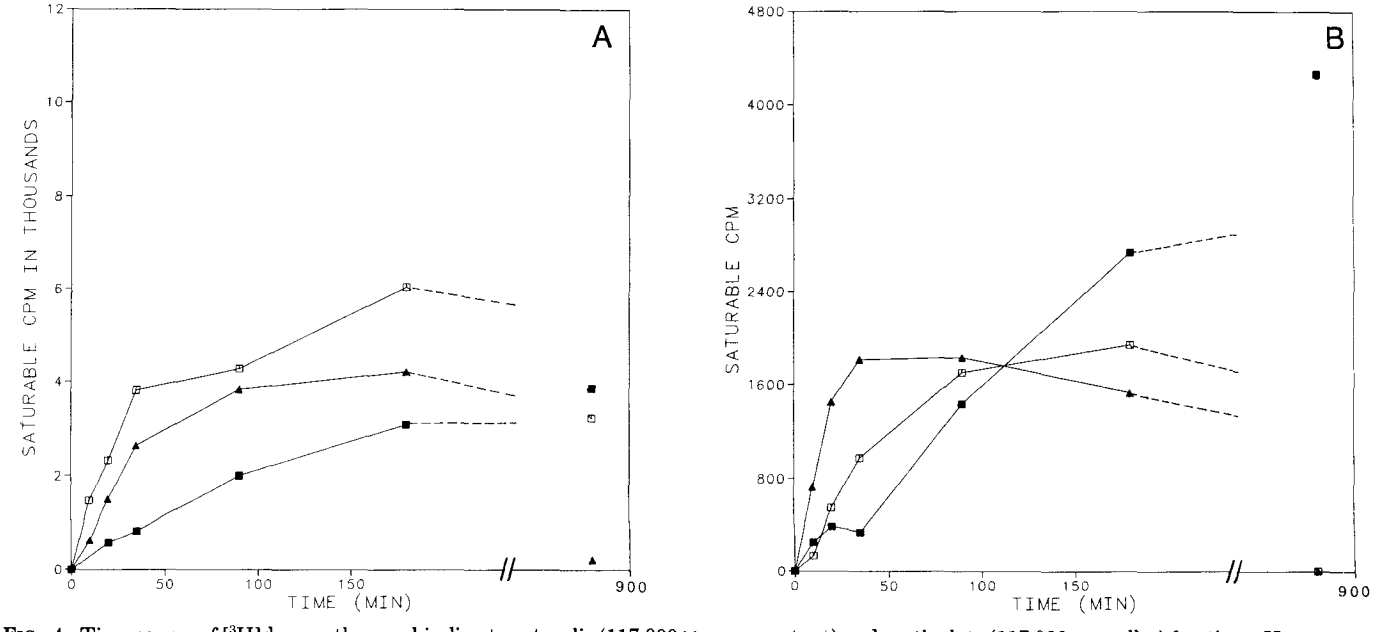

FIG. 4. Time course of [^{3}H]dexamethasone binding to cytosolic (117,000 × *g* supernatant) and particulate (117,000 × *g* pellet) fractions. Homogenate was incubated with 20 nM [^{3}H]dexamethasone in the presence and absence of a 1000-fold excess of unlabeled dexamethasone at 4 C (■—■), 20 C (□—□), and 30 C (▲—▲). Aliquots were removed at the indicated times up to 21 h, and the saturable radioactivity was determined. A, Cytosolic fraction. B, Particulate fraction.

TABLE 3. Effect of equilibration time on the $K_d$app and n for [^{3}H] dexamethasone in 117,000 × *g* supernatant and pellet fractions from rat liver

	$K_d$app (nM)	n (pmol/mg protein)
117,000 × *g* supernatant		
1.5 h	7.0	0.19
11 h	10	0.24
24 h	20	0.23
48 h	58	0.25
	μM	pmol/mg protein
117,000 × *g* pellet		
1.5 h	0.43	1.2
11 h	0.30	4.3
24 h	0.22	3.8
48 h	0.29	4.8

Incubation of 117,000 × *g* supernatant with [^{3}H]dexamethasone was performed as described in Fig. 5 with varied time of incubation, as indicated above. Incubation conditions for the 117,000 × *g* pellet are described in Fig. 6.

mechanism:

$$X \xrightarrow{k_1} Y \xrightarrow{k_2} I \quad \text{(IV)}$$

In this case k_2, the rate-limiting step, represents a first order production of *I*. Thus,

$$d[I]/dt = k_2[Y] \quad \text{(V)}$$

Y is produced from *X* in a zero order or pseudozero order mechanism.

$$d[Y]/dt = k_1 \quad \text{(VI)}$$

and

$$[Y] = k_1 t + c \quad \text{(VII)}$$

Substituting for [*Y*] in Eq V, the rate expression for the formation of *I* becomes:

$$d[I]/dt = k_1 k_2 t + k_2 c \quad \text{(VIII)}$$

This is of the same form as Eq III.

Thus, it appears that the data in Table 3 correlate extremely well with a first order release of a competitive inhibitor in the 117,000 × *g* supernatant. What the nature of such an inhibitor might be is unclear. However, the data in Table 3 suggest that $K_d$app calculated from incubations of shorter time periods are more representative of the actual K_d. Therefore, for 117,000 × *g* supernatant (cytosolic)-binding sites, an incubation time of 2 h at 4 C was used to minimize thermolability and achieve the best conditions approximating equilibrium. For 117,000 × *g* pellet binding, incubation conditions of 24 h at 4 C were used.

At increased concentrations of [^{3}H]dexamethasone, both soluble and particulate fractions demonstrated saturable [^{3}H]dexamethasone binding (Figs. 5 and 6). Lineweaver-Burk plots indicate homogeneous binding sites with vastly different binding affinities and number of binding sites. The binding affinity of [^{3}H]dexamethasone in the soluble 117,000 × *g* fraction was 7.2 nM (Fig. 5B), whereas an approximately 60-fold higher $K_d$app ($K_d$app = 0.42 μM; Fig. 6B) was calculated for the particulate fraction. In addition, the number of binding sites in the particulate fraction was 17-fold higher than that for cytosolic receptor. The $K_d$app for [^{3}H]cortisol binding to membrane fractions was also determined and was similar to that for [^{3}H]dexamethasone binding ($K_d$app = 0.18 μM; data not shown).

$K_d$app and n were also evaluated for adrenalectomized rat liver. In particulate fraction from adrenalectomized rat liver, no binding could be determined in rat liver obtained 4–6 days after adrenalectomy, while no change in $K_d$app or n was found for soluble receptor. However, after 20–30 days, membrane binding was reestablished. Table 4 shows the average of three individual determinations for particulate and soluble binding determined from both nonadrenalectomized and long term (20–30 days) postadrenalectomized rats. Similar $K_d$app values were found for soluble [^{3}H]dexamethasone binding from adrenalectomized rat liver ($K_d$app = 5.4 ± 0.64 nM) and nonadrenalectomized rat liver ($K_d$app = 7.9 ± 1.5 nM). The $K_d$app for particulate sites was 0.48 ± 0.084 μM in nonadrenalectomized animals and 0.44 ± 0.12 μM in long term adrenalectomized animals. The number of binding sites per mg protein in the pellet after 2-h incubation with [^{3}H]dexamethasone was 17-fold greater than that in the supernatant of nonadrenalectomized animals (5.7 ± 0.35 *vs.* 0.33 ± 0.044 pmol/mg protein). A 9-fold greater number of binding sites was found in long term adrenalectomized animals (3.5 ± 1.2 *vs.* 0.37 ± 0.16 pmol/mg protein). If normalized to absolute number of binding sites, a 9.1:1 ratio of particulate to soluble sites was found for nonadrenalectomized animals, and a 4.7:1 ratio was found for long term adrenalectomized animals.

Steroid specificity was evaluated for particulate fraction using [^{3}H]cortisol, which binds with higher affinity than [^{3}H]dexamethasone (Table 5). [^{3}H]Cortisol could be effectively competed by a 100-fold molar excess of unlabeled cortisol, dexamethasone, or progesterone. Testosterone and estrogen were less effective (48.0% and 74.9% of the control value, respectively), and androstanediol was ineffective (97.9% of the control value) in competing for [^{3}H]cortisol binding. It was concluded that the steroid-binding protein in rat liver membrane is best characterized as a glucocorticoid binder.

Discussion

The present studies identify specific glucocorticoid binding associated with particulate fractions of the rat

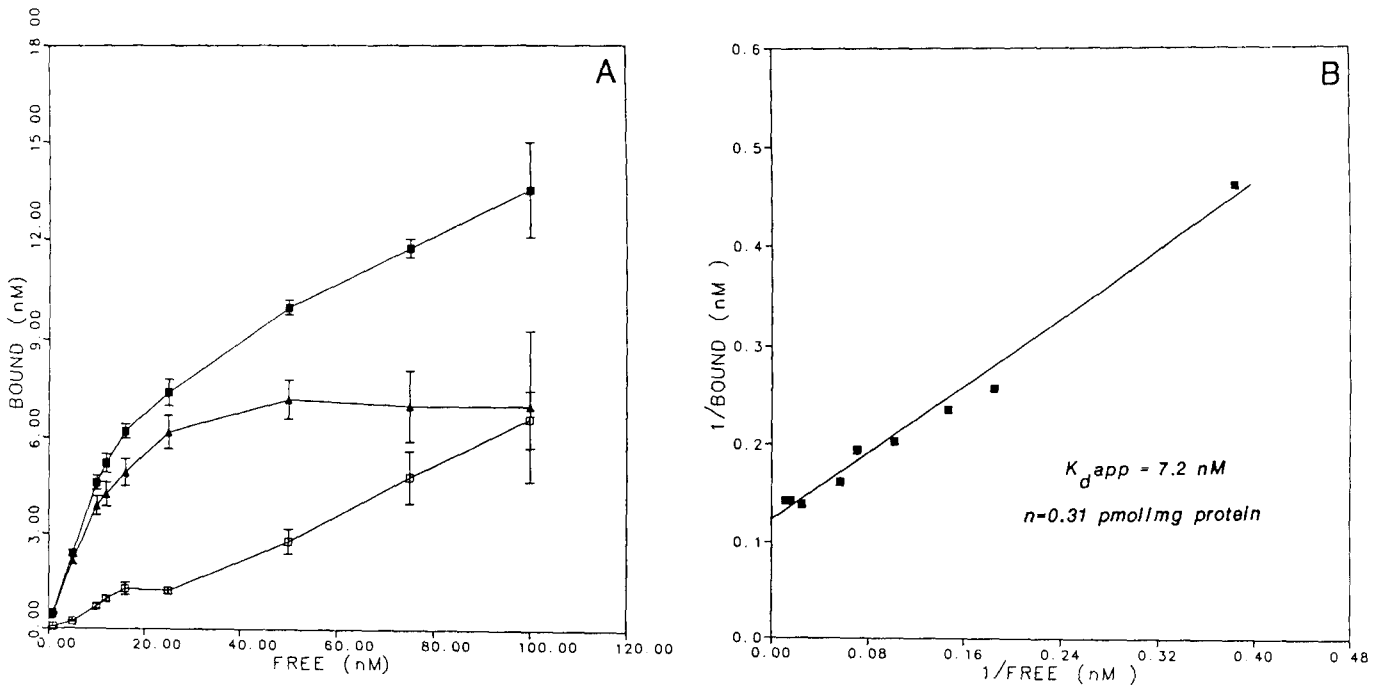

FIG. 5. A, Concentration dependence of [^{3}H]dexamethasone binding to cytosolic fraction. Rat liver homogenate was centrifuged at 117,000 × *g* to obtain supernatant (cytosolic fraction). Cytosolic fraction was incubated at 4 C within [^{3}H]dexamethasone at concentrations ranging from 2.63 nM to 0.10 μM, alone (■—■) or in the presence of 5 × 10^{-5} M unlabeled dexamethasone (□—□). The concentration of specifically bound [^{3}H] dexamethasone (▲—▲) was calculated as described in *Materials and Methods*. B, Lineweaver-Burk representation of the data for saturable bound [^{3}H]dexamethasone.

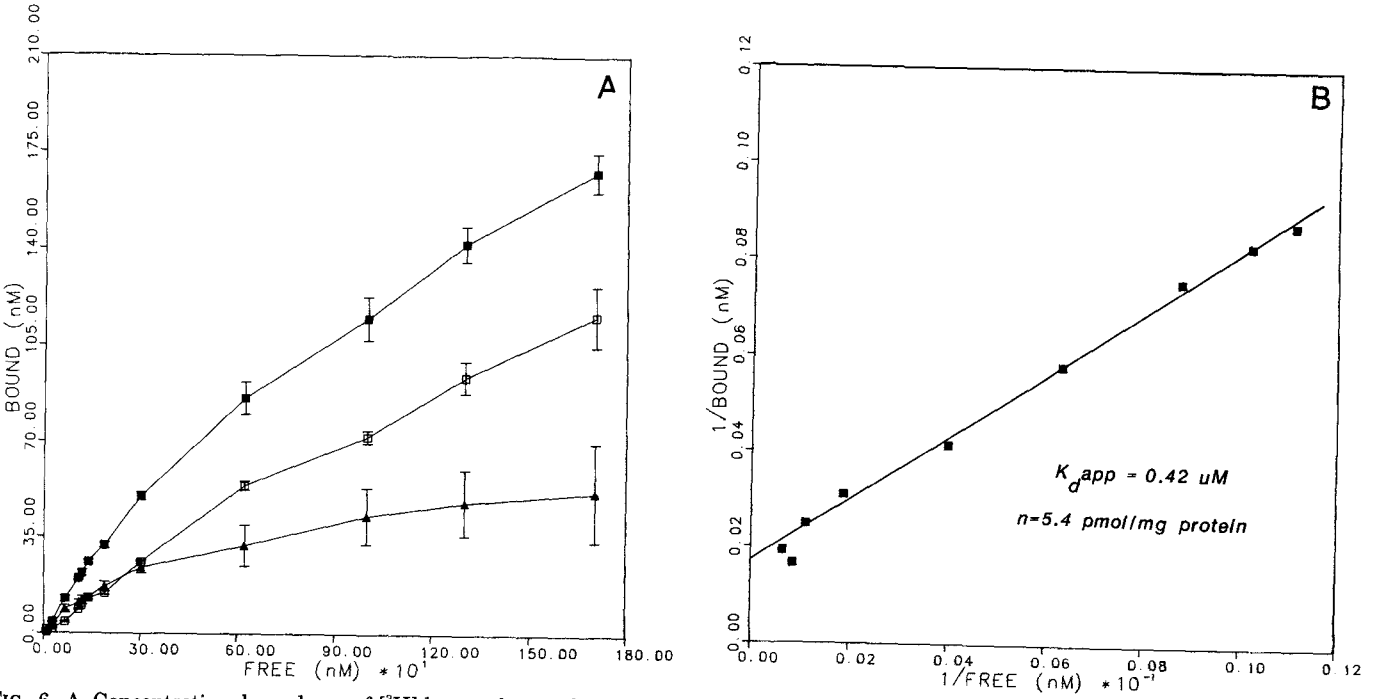

FIG. 6. A, Concentration dependence of [^{3}H]dexamethasone binding to particulate fraction. Rat liver homogenate was centrifuged at 117,000 × *g* to obtain pellet, which was resuspended to the original volume in MMM buffer. The pellet was incubated at 4 C with [^{3}H]dexamethasone at concentrations ranging from 9.0 nM to 1.7 μM, alone (■—■) or in the presence of 5 × 10^{-5} M unlabeled dexamethasone (□—□). The concentration of specifically bound [^{3}H]dexamethasone (▲—▲) was calculated as described in *Materials and Methods*. B, Lineweaver-Burk representation of the data for saturable bound [^{3}H]dexamethasone.

TABLE 4. Effect of adrenalectomy on the $K_d$app and n for [^{3}H]dexamethasone in 117,000 × *g* supernatant and pellet fractions from rat liver

	117,000 × *g* supernatant		117,000 × *g* pellet	
	$K_d$app (nM)	n (pmol/mg protein)	$K_d$app (μM)	n (pmol/mg protein)
Nonadrenalectomy	7.9 (1.5)	0.33 (0.044)	0.48 (0.084)	5.7 (0.35)
Adrenalectomy	5.4 (0.64)	0.37 (0.16)	0.44 (0.12)	3.5 (1.2)

Incubation of the 117,000 × *g* supernatant with [^{3}H]dexamethasone was performed as described in Fig. 5. Incubation of the 117,000 × *g* pellet with [^{3}H]dexamethasone was performed as described in Fig. 6. Adrenalectomized rats were bilaterally adrenalectomized 20–30 days before experimentation. Each number represents the average of three determinations (±SD).

TABLE 5. Steroid specificity of [^{3}H]cortisol binding to 117,000 × *g* pellet from nonadrenalectomized rats

Competing steroid[a]	Specific [^{3}H]cortisol binding	
	dpm/mg protein	% of control[b]
None	2390 (14.0)	100 (0.0)
Cortisol	90.1 (7.3)	0.0 (0.0)
Dexamethasone	452 (27.9)	15.7 (1.6)
Progesterone	87.5 (28.8)	0.0 (0.0)
Testosterone	1190 (15.2)	48.0 (0.91)
17β-Estradiol	1810 (73.8)	74.9 (3.5)
Androstanediol	2340 (78.2)	97.9 (3.8)

Each number represents the average of three determinations (±SD).

[a] 2×10^{-6} M.

[b] Difference in [^{3}H]cortisol binding for the 117,000 × *g* pellet incubated with 2×10^{-8} M [^{3}H]cortisol in the presence of unlabeled cortisol (2×10^{-6} M; control) or unlabeled competing steroid (2×10^{-6} M).

Only about 30% is associated with the soluble fraction, which contains cytosolic receptor. This indication of a predominance of plasma membrane steroid hormone-binding sites is significantly greater than has previously been reported for target cells shown to contain both particulate and soluble steroid hormone binding.

The $K_d$app for particulate sites (0.48 ± 0.084 μM) is much higher than that for soluble sites (7.9 ± 1.5 nM). Therefore, the normal conditions of incubation do not accurately represent actual cellular distribution, as the [^{3}H]dexamethasone concentration is significantly lower than the $K_d$app for plasma membrane sites and will preferentially label a greater percentage of the cytosolic sites. Particulate sites were determined by kinetic analysis to be at least 9.1-fold more concentrated than soluble cytosolic sites. However, these numbers are conservative. In the preparation of homogenate, a significant amount of particulate material is removed, accounting for 75–80% of the total cellular 5′-nucleotidase activity (data not shown) and presumably also particulate-bound steroid-binding macromolecules. A ratio as high as 45:1 may be closer to the actual cellular distribution.

The question of adventitious binding of the soluble binding sites to particulate fraction is discounted for several reasons. First, extensive sonication had little effect on particulate binding sites, suggesting that vesicle-trapped soluble receptor is not contributing significantly to the observed [^{3}H]dexamethasone binding. Secondly, radiolabeled membrane could be collected on GF/B filters and washed extensively without any decrease in saturable [^{3}H]dexamethasone binding. Thirdly, [^{3}H]dexamethasone-binding macromolecules in particulate fractions showed significant differences in binding affinity from soluble cytosolic receptor. Therefore, it does not seem likely that the plasma membrane binding sites represent artifactual association of cytosolic receptor to membrane in homogenate preparations.

Several other reports have supported the presence of high affinity steroid hormone binders in membrane preparations. Parikh *et al.* (20) found 7–15% of the total 17β-[^{3}H]estradiol-binding sites in microsomal fractions of the intact calf uteri. Using plasma membrane subfractions from hepatocytes to ovariectomized rats, Pietras and Szego (21) reported that about 44% of the estrogen-binding macromolecules were concentrated in the plasma membrane.

In a previous analysis of glucocorticoid-binding sites in rat liver plasma membrane, Suyemitsu and Terayama (22) noted similarities between cytosolic binders described by Beato and Feigelson (10) and membrane-associated binding sites identified in rat liver membrane preparations. Binding affinities ranging from 1.4–31 nM were determined for [^{3}H]cortisol, and the lower of these correlated with the cytosolic receptor ($K_d$app = 7.94 ± 1.5 nM). This affinity is considerably greater than the affinity of membrane fractions reported for [^{3}H]cortisol herein ($K_d$app = 0.18 μM). Furthermore, Suyemitsu and Terayama (22) showed that plasma membrane fractions had no affinity for [^{3}H]dexamethasone at concentrations up to 1 μM. As we have shown, this concentration is adequate to obtain observable binding in membrane sites, based on a $K_d$app of 0.48 ± 0.084 μM and should be sufficient for this kinetic analysis. However, the centrifugal method employed by Suyemitsu and Terayama for determination of [^{3}H]cortisol bound to membrane fractions does not employ sufficient centrifugation speed (17,000 × *g*; 20 min) to cause sedimentation of membrane fractions. This in particular could allow for the significant differences in K_d reported for [^{3}H]cortisol and allow for the lack of identification of specific binding sites for

[^{3}H]dexamethasone. Equilibrium dialysis was also used to estimate [^{3}H]cortisol binding. However, the extensive time required to attain equilibrium (48 h) was suggested by Beato and Feigelson (10) to have caused previous detection problems with cytosolic receptor in the same tissue.

In rat liver, a critical prerequisite to the identification of particulate glucocorticoid-binding macromolecules is the use of intact rather than adrenalectomized animals. Adrenalectomy was shown to directly affect the identification of particulate [^{3}H]dexamethasone-binding sites. The possibility exists that these binding sites are completely lost from the particulate fractions in the absence of circulating glucocorticoid or in some manner altered or inhibited from [^{3}H]dexamethasone binding. It appears that binding sites are regenerated to approximately two thirds of preadrenalectomy conditions (normal) by 20–30 days postadrenalectomy. In general, the use of nonorganectomized animals may favor the observation of particulate binding sites for steroid hormones. The prevalent use of organectomy may explain the relative paucity of published studies supporting their presence.

Our results provide evidence that the most abundant [^{3}H]dexamethasone binding in rat liver cells is associated with a plasma membrane-enriched fraction. The relationship of such binding to biological effects is unknown. It is possible that macromolecular membrane association of steroid hormone before micropinocytosis is a prerequisite for steroid hormone action (1). However, it is also possible that membrane association is not directly related to steroid hormone action. This mechanism of steroid binding may be designed for another nontranscriptional biological action, such as the rapid metabolism of steroid hormone. Since the binding affinity for both dexamethasone and the natural glucocorticoid cortisol is greater than that expected for the maximal response to circulating steroid concentration (normally in the range of 0.1 nM to 0.1 μM), it is possible that this mechanism of action is functional only at elevated steroid concentrations, for example in response to high glucocorticoid levels exemplary of stress conditions. Such action might elicit a specific response to elevated glucocorticoid levels or may simply reflect an increased attempt to metabolize steroid. Clearly, the presence of endogenous glucocorticoid is important, if not regulatory, to this process and cannot be overlooked in future work on this system.

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