Glucocorticoid–Receptor Interactions

STUDIES OF THE NEGATIVE CO-OPERATIVITY INDUCED BY STEROID INTERACTIONS WITH A SECONDARY, HYDROPHOBIC, BINDING SITE

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(Received 25 July 1979)

The effects of steroids on the binding of [1,2-³H]dexamethasone and [1,2-³H]progesterone to the glucocorticoid receptor of rat thymus cytosol were studied. Although both glucocorticoid agonists and antagonists competed with [1,2-³H]dexamethasone for binding to the receptor under equilibrium conditions, only glucocorticoid antagonists or partial agonists, at micromolar concentrations, were capable of accelerating the rate of dissociation of previously bound [1,2-³H]dexamethasone from the receptor. Antagonists or partial agonists also enhanced the rate of dissociation of [1,2-³H]progesterone from the glucocorticoid receptor, with identical specificity and concentration–response characteristics. These effects are attributed to the presence on the receptor of a secondary, low-affinity, binding site for glucocorticoid antagonists, the occupancy of which produces negatively co-operative interactions with the primary glucocorticoid-binding site. In contrast with the interactions with the primary site, the interactions of steroids with the negatively co-operative site appear to be primarily hydrophobic in nature, and the site resembles the steroid-binding site of progestin-binding proteins in its specificity, though not its affinity. The results also suggest that the initial interactions of both glucocorticoid agonists and antagonists with the receptor under equilibrium conditions are with one primary site on a receptor existing in one conformation only.

Studies of the mechanism of action of glucocorticoids (reviewed by Munck & Leung, 1977) have revealed that the initial steps in the action of such compounds include their interaction with receptor proteins in the soluble cytosol fraction of target cells, followed by a temperature-dependent activation process that results in the steroid–receptor complex exhibiting an increased affinity for nuclear binding sites. Partial or complete anta-

gonism of the biological actions of active glucocorticoids by structurally related steroids such as progesterone or 11-deoxycortisol (cortexolone) has been demonstrated both *in vivo* (Desser-Wiest & Desser, 1977; Duncan & Duncan, 1979) and *in vitro* (Makman *et al.*, 1967; Munck & Brinck-Johnsen, 1968; Samuels & Tomkins, 1970; Dausse *et al.*, 1977). Glucocorticoid antagonists compete with biologically active steroids such as dexamethasone for binding to the non-activated form of the receptor (Rousseau *et al.*, 1972; Jones *et al.*, 1979), but appear to have no or only limited ability to stimulate the cytoplasmic depletion and nuclear accumulation of the receptor (Rousseau *et al.*, 1973; Wira & Munck, 1974; Turnell *et al.*, 1974; Kaiser *et al.*, 1979), suggesting that pure antagonists are incapable of promoting receptor activation, and that partial antagonists may do so only to the extent that they are also glucocorticoid agonists.

Although such studies appear to localize the site of discrimination between agonists and antagonists at the level of steroid interactions with the cytosol

Trivial and systematic names of steroids mentioned in the text: cortisol, 11 β ,17,21-trihydroxypregn-4-ene-3,20-dione; 11-deoxycorticosterone, 21-hydroxypregn-4-ene-3,20-dione; 11-deoxycortisol, 17,21-dihydroxypregn-4-ene-3,20-dione; dexamethasone, 9 α -fluoro-16 α -methyl-11 β ,17,21-trihydroxypregna-1,4-diene-3,20-dione; 17-hydroxyprogesterone, 17-hydroxypregn-4-ene-3,20-dione; norethisterone, 17 α -ethinyl-17 β -hydroxy-19-norandrost-4-en-3-one; progesterone, pregn-4-ene-3,20-dione; compound R5020, 17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione; triamcinolone acetonide, 11 β ,16 α -17,21-tetrahydroxy-9 α -fluoropregna-1,4-diene-3,20-dione 16 α ,17-acetonide.

receptor, the mechanism of discrimination remains obscure. Observations that progesterone exhibits a higher rate of association with the receptor than does dexamethasone, and that progesterone-receptor complexes are more susceptible to thermal inactivation than are dexamethasone-receptor complexes (Rousseau *et al.*, 1972), have been interpreted as supporting a two-state allosteric model (Monod *et al.*, 1965; Samuels & Tomkins, 1970), in which antagonists interact with an inactive conformation of the receptor that predominates in the absence of steroid, whereas agonists interact with a minor active conformation, thus increasing its concentration. The data can, however, also be fitted to an induced-fit type of model (Koshland & Neet, 1968; Pratt *et al.*, 1975), and neither model specifies the nature of the steroid-binding site or sites.

Suthers *et al.* (1976) have reported that glucocorticoid antagonists and partial agonists, such as progesterone and 11-deoxycorticosterone, but not pure agonists, can increase the rate at which dexamethasone dissociates from the glucocorticoid receptor of rat liver and kidney at 25°C. They concluded that the receptor possesses two binding sites, one for agonists and one for antagonists, and that binding of one class of steroid diminishes the affinity of the other site for its respective class of steroid. Since these experiments were performed at 25°C, a temperature at which receptor activation occurs, it was possible that the increased rate of dissociation reflected properties of the activated form of the receptor. In the present work, this phenomenon was studied at 0°C under conditions of minimal receptor activation. Under such conditions, a number of steroids, including progesterone, were observed to enhance the rate of dissociation of glucocorticoid agonists such as dexamethasone from the receptor. The site with which these steroids interact to promote dissociation has a steroid-specificity similar to that of progestin receptors, but is of low affinity. The same steroids also enhance the dissociation of progesterone from the glucocorticoid receptor, with identical specificity and affinity characteristics, suggesting that antagonists, at low concentrations, and agonists bind to the same receptor site on the same receptor conformation.

Experimental

Materials

[1,2-³H]Dexamethasone (25 Ci/mmol), [1,2-³H]progesterone (49 Ci/mmol) and [1,2,4-³H]triamcinolone acetonide (20 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Non-radioactive steroids were from Sigma (London) Chemical Co. (Poole, Dorset, U.K.), Steraloids (Wilton, NH, U.S.A.) or Roussel-Uclaf (Romainville, France). Neutralized acti-

vated charcoal, dithiothreitol and bovine serum albumin were also from Sigma; Sephadex G-25 and Dextran T-70 were from Pharmacia Fine Chemicals (London W.5, U.K.). All other reagents were obtained from BDH Chemicals (Poole, Dorset, U.K.).

Preparation of rat thymus cytosol

Thymus tissue was obtained from 8–12-week-old male Sprague-Dawley rats, bred in the Institute Animal Colony and adrenalectomized 1–2 weeks before use. Animals were killed by decapitation under light ether anaesthesia. Thymus tissue was excised and homogenized in 10 vol. of TEDKM buffer (1 mM-EDTA, 2.5 mM-dithiothreitol, 25 mM-KCl and 10 mM-Na₂MoO₄ in 50 mM-Tris/HCl buffer, pH 7.4 at 20°C) at 0–4°C with ten strokes of a motor-driven Teflon pestle in a glass homogenizer. The homogenate was centrifuged at 114 000 *g* for 30 min at 4°C; the supernatant (cytosol) fraction, containing 3–4 mg of protein/ml, was removed free of floating fat and used immediately.

Steroid-binding studies

Radioactive steroids were added to cytosol as solutions in TEDKM buffer. Non-radioactive steroids were prepared as stock solutions in ethanol, diluted with TEDKM buffer (19:1, v/v) and added to incubations in volumes such that the final concentration of ethanol was 1% (v/v); this concentration of ethanol did not affect steroid-binding parameters. Control incubation mixtures received an equivalent amount of ethanol-containing buffer alone. After incubation of cytosol with steroids at 0°C, protein-bound radioactive steroid was determined by chromatography on columns (bed volume 9.1 ml) of Sephadex G-25 or by dextran/charcoal competitive binding assay, as described previously (Rees & Bell, 1975). For the latter assay, portions of steroid-treated cytosol were incubated at 0°C with an equivalent volume of a suspension of neutralized activated charcoal (20 g/l) and Dextran T-70 (2 g/l) in 50 mM-Tris/HCl buffer, pH 7.4 at 20°C. After centrifugation at 800 *g* for 10 min, portions of the supernatant fraction were taken for counting of radioactivity. The time of incubation with dextran/charcoal varied between 5 and 20 min, depending on the radioactive ligand used; results were corrected for dissociation during the assay as described previously (Rees & Bell, 1975). To assess specific, saturable, glucocorticoid binding, parallel incubations of cytosol with radioactive steroids were performed in the presence and absence of 10 μM-dexamethasone; the difference in the amounts of protein-bound steroid between the two incubations was taken as specific binding.

Sucrose-density-gradient centrifugation of steroid-receptor complexes

Rat thymus cytosol in TEDKM buffer was incubated for 4.5 h at 0°C with 20 nM-[1,2,4-³H]triamcinolone acetone. Free steroid was then removed by incubation with the pellet centrifuged from an equivalent volume of dextran/charcoal suspension, for 20 min at 0°C, followed by centrifugation at 800g for 10 min. Samples of the supernatant fraction were applied to linear 5–20% (w/v) sucrose density gradients, prepared in TEDKM buffer and containing 50 μM-cortisol or 50 μM-progesterone. Gradients were centrifuged at 114 000g for 18 h at 4°C, and subsequently fractionated by upward displacement with 40% (w/v) sucrose and analysed for radioactivity. Bovine serum albumin ($s_{20,w}$ 4.6S) was applied to parallel gradients as a sedimentation marker, and located by its absorbance at 280 nm.

Measurement of radioactivity

Radioactivity was measured for aqueous samples (0.5 ml) in 10 ml of liquid scintillant composed of 2,5-diphenyloxazole (5 g) in toluene (1000 ml) and Triton X-100 (500 ml) by using a Nuclear-Chicago mark 2 liquid-scintillation spectrometer. The counting efficiency was 30–35%; corrections for quenching were made by the channels-ratio method by using calibration curves for ³H.

Results

Steroid binding at equilibrium

In preliminary experiments it was established that the time taken for the specific binding of [1,2-³H]dexamethasone to the rat thymus glucocorticoid receptor to reach steady-state values at 0°C varied between 4 and 24 h over the steroid concentration range used (0.1–50 nM). The half-life of the free receptor under the conditions employed was about 9–10 days (Bell & Jones, 1979a), sufficient for the steady-state binding values to represent a close approximation to true equilibrium values. Saturation analysis of the interaction between [1,2-³H]dexamethasone and the receptor after incubation at 0°C for 24 h indicated that specific binding was to a single class of sites with $K_d = 4.69 \pm 0.87$ nM (mean $\pm$ S.E.M., $n = 3$). The specific binding of [1,2-³H]dexamethasone could be inhibited by a variety of other steroids, including compounds known to be pure or partial glucocorticoid agonists or glucocorticoid antagonists (Munck & Brinck-Johnsen, 1968; Samuels & Tomkins, 1970). The data in all cases were compatible with competitive inhibition of [1,2-³H]dexamethasone binding, and there was no evidence for second-site binding by any of the compounds

studied, including progesterone (Fig. 1), at concentrations up to 1 μM. Inhibition constants for a range of steroids, relative to dexamethasone, are given in Table 1. Fig. 2 shows the effect of 1 μM-progesterone on the equilibrium binding of [1,2-³H]dexamethasone, analysed by the Hill plot (Barcroft & Hill, 1909); plots were linear both in the absence and presence of progesterone, with slopes of 0.991 and 0.980 respectively, and the only effect of progesterone was to change the apparent K_d for [1,2-³H]dexamethasone from 3.79 to 161 nM.

Dissociation of [1,2-³H]dexamethasone from the receptor

The dissociation of [1,2-³H]dexamethasone from the rat thymus glucocorticoid receptor at 0°C was

Fig. 1. Competitive inhibition of [1,2-³H]dexamethasone binding

Cytosol was incubated for 24 h at 0°C with 20 nM-[1,2-³H]dexamethasone and the indicated concentrations of dexamethasone (●) or progesterone (■). Values for specific binding in the presence of the competitors (B) were normalized relative to control values (B_0).

Fig. 2. Hill plots of [1,2-³H]dexamethasone binding data. Cytosol was incubated for 24 h at 0°C with various concentrations of [1,2-³H]dexamethasone in the absence (●) or presence (■) of 1 μM-progesterone, and the concentration of specifically bound [1,2-³H]dexamethasone (B) was determined.

Table 1. Steroid effects on the equilibrium binding and dissociation of [1,2-³H]dexamethasone
K_i values were obtained from the slopes of normalized reciprocal plots similar to those shown in Fig. 1. Steroid effects on dissociation were determined after a 5 h dissociation phase initiated by the addition of 1 μM-dexamethasone together with individual steroids at 50 μM to cytosol previously incubated to equilibrium at 0°C with 20 nM-[1,2-³H]-dexamethasone. Results are expressed relative to the values obtained with dexamethasone alone, and their significance was assessed with Student's *t* test (N.S., not significant).

Steroid	Relative <i>K_i</i>	[1,2- ³ H]Dexamethasone undissociated (% ± S.D., <i>n</i> = 4)	2 <i>P</i>
Pregn-4-ene-3,20-dione (progesterone)	8.41	61.2 ± 2.7	3.5 × 10 ⁻⁶
21-Hydroxypregn-4-ene-3,20-dione (11-deoxycorticosterone)	3.94	72.4 ± 2.1	1.8 × 10 ⁻⁵
6α,16α-Dimethylpregn-4-ene-3,20-dione	1.15	75.8 ± 3.8	1.2 × 10 ⁻⁴
17β-Hydroxyandrost-4-en-3-one (testosterone)	660	87.3 ± 0.9	1.0 × 10 ⁻³
Androst-4-ene-3,17-dione	1760	88.4 ± 1.9	1.9 × 10 ⁻³
11β-Hydroxypregn-4-ene-3,20-dione	3.66	89.8 ± 2.5	4.8 × 10 ⁻³
16α-Methylpregn-4-ene-3,20-dione	8.13	88.7 ± 3.6	5.6 × 10 ⁻³
17,21-Dihydroxypregn-4-ene-3,20-dione	47.5	91.7 ± 1.9	9.7 × 10 ⁻³
17β-Hydroxy-17α-methylandrost-4-en-3-one	317	91.1 ± 3.0	1.2 × 10 ⁻²
Pregn-4-ene-3,11,20-trione	358	93.2 ± 2.1	2.3 × 10 ⁻²
11α-Hydroxypregn-4-ene-3,20-dione	256	96.0 ± 0.9	N.S.
11β,17,21-Trihydroxypregn-4-ene-3,20-dione (cortisol)	18.2	95.4 ± 3.5	N.S.
11β,17-Dihydroxypregn-4-ene-3,20-dione	33.8	96.8 ± 1.7	N.S.
17-Hydroxypregn-4-ene-3,20-dione	91.3	96.7 ± 5.1	N.S.
11β,21-Dihydroxypregn-4-ene-3,20-dione (corticosterone)	3.00	97.0 ± 4.4	N.S.
17-Hydroxy-6α-methylpregn-4-ene-3,20-dione	3.49	97.8 ± 2.1	N.S.
17α-Hydroxyandrost-4-en-3-one	1340	97.9 ± 3.0	N.S.
9α-Fluoro-16α-methyl-11β,17,21-trihydroxypregna-1,4-diene-3,20-dione (dexamethasone)	1.00	100.0 ± 4.0	—

Fig. 3. Steroid effects on the dissociation of [1,2-³H]dexamethasone and [1,2-³H]progesterone
(a) After incubation of cytosol with 20 nM-[1,2-³H]dexamethasone for 4.5 h at 0°C, free steroid was removed by gel exclusion chromatography. Then 50 μM-dexamethasone (●), 50 μM-progesterone (■) or buffer alone (▲) was added, and incubation was continued at 0°C. Samples were withdrawn for determination of bound [1,2-³H]dexamethasone at the times indicated. (b) Cytosol was incubated with 20 nM-[1,2-³H]progesterone for 4.5 h at 0°C, then dissociation was initiated by the addition of 50 μM-dexamethasone (●) or 50 μM-progesterone (■). Samples were withdrawn at intervals for the determination of bound [1,2-³H]progesterone.

first studied by removal of free [1,2-³H]dexamethasone. After incubation of cytosol with 20 nM-[1,2-³H]dexamethasone for 4.5 h at 0°C, free [1,2-³H]dexamethasone was removed by chromato-

graphy on Sephadex G-25 at 0–4°C. Dexamethasone or progesterone, to final concentrations of 50 μM, or buffer alone was then added, and the specific binding of [1,2-³H]dexamethasone was

determined after various periods of incubation at 0°C by dextran/charcoal competitive binding assay. The results (Fig. 3a) indicated that the rate of dissociation of [1,2-³H]dexamethasone was unaffected by the presence of non-radioactive dexamethasone, but was markedly enhanced in the presence of 50 μ M-progesterone. Dissociation followed first-order kinetics in all cases, with rate constants for dissociation at 0°C of $1.85 \times 10^{-3} \text{ min}^{-1}$ in buffer alone, $1.81 \times 10^{-3} \text{ min}^{-1}$ in the presence of 50 μ M-dexamethasone and $4.82 \times 10^{-3} \text{ min}^{-1}$ in the presence of 50 μ M-progesterone.

In further experiments, the dissociation of [1,2-³H]dexamethasone was initiated by displacement with various concentrations of non-radioactive dexamethasone or progesterone, singly or in combination, and the concentration of [1,2-³H]dexamethasone remaining bound was determined after 5 h by dextran/charcoal competitive binding assay. Specific binding in control incubations to which no non-radioactive steroid had been added declined by only 3% over this period, and this decline could be attributed entirely to dilution effects. The results obtained are shown in Fig. 4(a). At concentrations of non-radioactive dexamethasone in the range 1–50 μ M, sufficient to compete effectively with [1,2-³H]dexamethasone for association to the receptor, the rate constant for dissociation for [1,2-³H]dexamethasone showed little change, with a plateau value of $1.87 \times 10^{-3} \text{ min}^{-1}$ being reached at 10 μ M-dexamethasone. A dexamethasone concentration of 100 nM, representing only a 5-fold excess over the concentration of [1,2-³H]dexamethasone, was insufficient to block the association process entirely, and gave a low apparent dissociation rate constant. This phenomenon was even more apparent in the presence of low concentrations of progesterone. As the concentration of progesterone was increased beyond 1 μ M, however, the rate constant for dissociation for [1,2-³H]dexamethasone first attained the value seen in the presence of dexamethasone and then progressively increased, attaining a value of $4.63 \times 10^{-3} \text{ min}^{-1}$ in the presence of 50 μ M-progesterone. Higher concentrations of these steroids could not be tested owing to problems of solubility. The enhancing effect of progesterone on the dissociation rate was also observed in the presence of 10 μ M-dexamethasone, although the incomplete effect observed at low concentrations was eliminated.

The steroid-specificity of this effect on the rate of dissociation of [1,2-³H]dexamethasone was also investigated. After incubation of rat thymus cytosol for 4.5 h at 0°C with 20 nM-[1,2-³H]dexamethasone, steroids were added at final concentrations of 50 μ M, together with 1 μ M-dexamethasone to compensate for any lack of effect of weak competitors on the association of [1,2-³H]dexamethasone. Specifically

Fig. 4. Concentration-dependence of steroid effects on the dissociation of [1,2-³H]dexamethasone and [1,2-³H]progesterone

Cytosol was incubated with (a) 20 nM-[1,2-³H]dexamethasone or (b) 20 nM-[1,2-³H]progesterone for 4.5 h at 0°C, then dissociation was initiated by displacement with the indicated concentrations of dexamethasone (●), progesterone (■) or progesterone and 10 μ M-dexamethasone (□). Bound [1,2-³H]dexamethasone was determined after a further 5 h, and bound [1,2-³H]progesterone after a further 15 min, at 0°C, and the apparent rate constants for dissociation (k_{-1}) were calculated.

bound [1,2-³H]dexamethasone was determined after a further 5 h at 0°C by dextran/charcoal competitive binding assay and expressed as a percentage of the amount measured with 51 μ M-dexamethasone present (Table 1). Of the steroids tested, progesterone and 11-deoxycorticosterone produced the largest effects, whereas active glucocorticoids such as cortisol and corticosterone were without effect. The synthetic progestins compound R5020 and norethisterone also influenced the rate of dissociation of [1,2-³H]dexamethasone, but their limited solubility precluded accurate quantification of the magnitude of the effects (results not shown).

Dissociation of [1,2-³H]progesterone

Since progesterone competed with dexamethasone for binding to the glucocorticoid receptor and was also capable of enhancing the rate of dissociation of dexamethasone from the receptor, it was decided to investigate the effect of the addition of other steroids on the rate of dissociation of [1,2-³H]progesterone from the glucocorticoid receptor. In

experiments analogous to those performed with [1,2-³H]dexamethasone, rat thymus cytosol was incubated for 4.5 h at 0°C with 20 nM [1,2-³H]-progesterone. Then 50 μM-dexamethasone, 50 μM-progesterone or buffer alone was added and the specific binding of [1,2-³H]progesterone was determined at intervals by dextran/charcoal competitive binding assay. The results, shown in Fig. 3(b), indicate that the rate of dissociation in the presence

Fig. 5. Combined concentration-response curves for the enhancement of the dissociation of [1,2-³H]dexamethasone and [1,2-³H]progesterone

Cytosol was incubated with 20 nM [1,2-³H]dexamethasone (● and ○) or 20 nM [1,2-³H]progesterone (■ and □) for 4.5 h at 0°C, then dissociation was initiated by the addition of the indicated concentrations of dexamethasone together with 50 μM-progesterone (○ and □) or progesterone together with 50 μM-dexamethasone (● and ■). Bound [1,2-³H]progesterone was determined after a further 15 min and bound [1,2-³H]dexamethasone after a further 5 h at 0°C, and apparent rate constants for dissociation (k_{-1}) were calculated.

Table 2. Steroid effects on the dissociation of [1,2-³H]progesterone

Steroid effects on dissociation were determined after a 10 min dissociation phase initiated by the addition of steroids at 50 μM to cytosol previously incubated for 2 h at 0°C with 20 nM [1,2-³H]progesterone. Results are expressed relative to the binding in the presence of 50 μM-dexamethasone, and their significance was assessed with Student's *t* test (N.S., not significant).

Steroid	[1,2- ³ H]Progesterone undissociated (% ± s.d., n = 2)	2P
Pregn-4-ene-3,20-dione (progesterone)	50.8 ± 3.6	3.4 × 10 ⁻³
21-Hydroxypregn-4-ene-3,20-dione (11-deoxycorticosterone)	70.2 ± 2.6	5.8 × 10 ⁻³
11β-Hydroxypregn-4-ene-3,20-dione	84.9 ± 1.2	1.0 × 10 ⁻²
17-Hydroxypregn-4-ene-3,20-dione	91.9 ± 0.9	3.1 × 10 ⁻²
17,21-Dihydroxypregn-4-ene-3,20-dione	93.0 ± 1.3	4.7 × 10 ⁻²
11β,21-Dihydroxypregn-4-ene-3,20-dione (corticosterone)	97.4 ± 0.5	N.S.
11β,17-Dihydroxypregn-4-ene-3,20-dione	98.9 ± 1.4	N.S.
11β,17,21-Trihydroxypregn-4-ene-3,20-dione (cortisol)	99.6 ± 1.2	N.S.
9α-Fluoro-16α-methyl-11β,17,21-trihydroxypregna-1,4-diene-3,20-dione (dexamethasone)	100.0 ± 1.8	—

of non-radioactive progesterone was considerably higher than in the presence of non-radioactive dexamethasone; the respective rate constants at 0°C were 0.028 and 0.055 min⁻¹. Dose-response curves for the effects of dexamethasone and progesterone on the rate of dissociation of [1,2-³H]progesterone (Fig. 4b) confirmed that progesterone enhanced its own rate of dissociation and indicated that dexamethasone had no such effect.

The similarities between the dose-response curves for the effects of progesterone on the dissociation of [1,2-³H]dexamethasone and [1,2-³H]progesterone were confirmed in an experiment in which the effects of dexamethasone and progesterone together on the dissociation of both radioactive steroids from the glucocorticoid receptor were investigated simultaneously. The precise parallelism in the dose-response curves obtained (Fig. 5) suggests that the same form of the receptor is involved in the interactions observed with both steroids. In confirmation of this conclusion, the steroid-specificity of the enhancement of the dissociation of [1,2-³H]progesterone was almost identical with that observed for [1,2-³H]dexamethasone, the only difference being that a marginal effect was observed with 17-hydroxyprogesterone (Table 2).

Sucrose-density-gradient centrifugation

To investigate whether the binding of progesterone to the enhancing site influenced the sedimentation properties of the steroid-receptor complex, portions of rat thymus cytosol containing the glucocorticoid receptor complexed with [1,2-³H]-triamcinolone acetonide were analysed by centrifugation in 5–20% (w/v) sucrose density gradients containing either 50 μM-cortisol or 50 μM-progesterone. No differences in the sedimentation profiles were observed; the radioactively labelled complex sedimented as a major peak with a sedimentation coefficient of approx. 6S and with a shoulder at approx. 8S in both gradients (Fig. 6).

Discussion

The analysis of the primary process of glucocorticoid-receptor interaction in crude subcellular fractions can be made more difficult by the simultaneous occurrence of other processes such as receptor activation and inactivation. The impact of these factors was minimized in the present investigations by the use of a low temperature (0°C) and by the presence of molybdate in the incubation media. In addition to stabilizing the receptor against inactivation (Nielsen *et al.*, 1977; Bell & Jones, 1979a), molybdate has been reported to inhibit receptor activation (Mierendorf & Mueller, 1979). Receptor activation is in any case slow at 0°C (Milgrom *et al.*, 1973). Aranyi (1979) has drawn attention to the difficulties of calculating true equilibrium constants for systems with slow equilibration and concomitant loss of one of the binding components, but the degree of stabilization of the receptor afforded by the use of molybdate permitted relatively long incubation times (approx. 5 times the *t*_{1/2} for dissociation of the complex) to be used, so that the system was close to true equilibrium. Under such conditions the binding process appeared to be a simple bimolecular association of the steroid with a single class of sites, with no evidence for cooperativity in the binding of [1,2-³H]dexamethasone even in the presence of the glucocorticoid antagonist progesterone. The experimentally derived equilibrium dissociation constant (4.69 nM) was similar to the quotient of rate constants (2–3 nM; T. R. Jones & P. A. Bell, unpublished work), suggesting that the system was indeed close to true equilibrium.

Studies of the inhibition of [1,2-³H]dexamethasone binding by other steroids, performed under identical conditions, indicated that both agonists and antagonists competed with the labelled ligand by binding either to the same site or to some mutually exclusive site. No evidence for the interaction of either agonists or antagonists with a second binding site was obtained over the concentration ranges tested (up to 0.1–1.0 μM for different steroids). The

Fig. 6. Sucrose-density-gradient centrifugation of [1,2,4-³H]triamcinolone acetonide-receptor complexes

Cytosol containing [1,2,4-³H]triamcinolone acetonide-receptor complexes was sedimented on 5–20% sucrose gradients containing 50 μM-cortisol (-----) or 50 μM-progesterone (—). Gradients were centrifuged for 18 h at 114 000 *g*, then fractionated by upward displacement. The arrow denotes the position of the sedimentation marker (bovine serum albumin, *s*_{20,w} 4.6 S).

structure-activity relationships for competitive inhibition were similar to those reported previously (Rousseau & Schmit, 1977; Wolff *et al.*, 1978; Jones *et al.*, 1979), with a marked dependence on the presence of polar groups at positions 11 β and 21 and non-polar groups at positions 6 α and 16 α for the enhancement of binding affinity.

Although all the steroids tested appeared to behave in a qualitatively similar manner in competitive binding studies at equilibrium, marked differences were observed in their effects on the dissociation of [1,2-³H]dexamethasone from the previously formed complex. The addition of up to 50 μ M-dexamethasone had no effect on the dissociation of [1,2-³H]dexamethasone, but the addition of 50 μ M-progesterone resulted in a 2.7-fold increase in the dissociation rate constant. The enhancing effect of progesterone on the dissociation rate of [1,2-³H]dexamethasone was concentration-dependent; a complete dose-response curve could not be obtained for progesterone because of its limited solubility, but an equilibrium dissociation constant in the range 50–100 μ M could be inferred from the data. The interaction of steroids with this second site, as evidenced by the enhancement of dissociation, displayed marked structural specificity, despite its relatively weak nature. Unlike the interaction with the primary binding site studied under equilibrium conditions, the interaction of steroids with the second site followed the polarity rule (Westphal, 1971), suggesting that the interaction was primarily hydrophobic. Furthermore, the enhancement of the rate of dissociation of [1,2-³H]dexamethasone was produced only by steroids that have been classified (Rousseau & Schmit, 1977) as glucocorticoid antagonists or partial agonists, and not by pure agonists. Interestingly, the steroid-binding characteristics of the enhancing site were similar to those of the plasma progesterin-binding globulin (Westphal, 1978) and the tissue progesterin receptor (Smith *et al.*, 1974; Walters & Clark, 1977; Vu Hai & Milgrom, 1978), suggesting the possibility of some degree of overlap between gestational and anti-glucocorticoid activities.

The enhancement of the rate of dissociation of agonists from glucocorticoid receptors by compounds such as progesterone has been studied in other systems (Suthers *et al.*, 1976; Yeakley & Harrison, 1979), although the steroid-specificity of the effect has been examined less extensively. To explain their observations, Suthers *et al.* (1976) proposed a model in which the receptor possessed two interacting binding sites, one specific for glucocorticoid agonists and the other for antagonists, and further suggested that the binding of one class of steroid would result in a conformational change in the receptor that would diminish the affinity of the other site for its respective class of

steroid. Such a model could be either of the allosteric (Monod *et al.*, 1965) or induced-fit form (Koshland & Neet, 1968). However, in the present work it was observed that the rate of dissociation of [1,2-³H]progesterone from the glucocorticoid receptor could be modified to a parallel extent by precisely the same steroids that enhanced the rate of dissociation of [1,2-³H]dexamethasone. Such behaviour would not be expected in terms of the model proposed by Suthers *et al.* (1976); rather, the rate of dissociation of antagonists such as progesterone should be enhanced by agonists, such as dexamethasone. Furthermore, a precise correspondence between negative co-operative effects on the binding of [1,2-³H]dexamethasone and [1,2-³H]progesterone would not be expected if the binding of agonists and antagonists were to mutually exclusive sites on different conformations of the receptor (Rousseau *et al.*, 1972), since the conformational changes might be expected to affect the negatively co-operative interactions between the primary and secondary binding sites. The observations suggest instead that the primary interactions of glucocorticoid agonists and antagonists with the receptor, which can be detected by direct or competitive equilibrium binding studies, are with a single class of sites on a receptor that exists in only one conformation, though subsequent conformational changes, perhaps related to receptor activation, cannot be ruled out (Bell & Jones, 1979b). Further kinetic studies are required to confirm these conclusions.

Irrespective of the mechanisms involved, the present results demonstrate the presence on the glucocorticoid receptor of two distinct classes of binding sites, with negatively co-operative interactions between them. The occupancy of the second, hydrophobic, steroid-binding site did not appear to influence the sedimentation properties of the receptor. Negative co-operativity has also been observed in the binding of insulin analogues to the lymphocyte insulin receptor, but with most insulin analogues a close correspondence was observed between the analogue concentrations that inhibited insulin binding and those that promoted the accelerated dissociation of insulin (De Meyts *et al.*, 1978). By contrast, there was an approximately 1000-fold difference between the progesterone concentrations that inhibited dexamethasone binding at equilibrium and those that enhanced the dissociation of dexamethasone. This concentration difference accounts for the failure to detect the second binding site in competition studies at equilibrium, and further indicates that the phenomenon is unlikely to be of any physiological significance. Nevertheless it may be of considerable mechanistic significance, for the observation that negative co-operativity can be evoked by glucocorticoid antagonists and partial

agonists but not by pure agonists suggests that the phenomenon is closely linked with the ability of the receptor to discriminate between agonists and antagonists, perhaps by virtue of the secondary, negatively co-operative, site functioning as an 'entry site' linked to the primary steroid-binding site (Bell & Jones, 1979b).

The authors thank the Tenovus Organization for continuing financial support.

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