Glucocorticoid–receptor interactions

Discrimination between glucocorticoid agonists and antagonists by means of receptor-binding kinetics

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The kinetics of binding to the molybdate-stabilized glucocorticoid receptor of rat thymus cytosol were determined at 0°C for a number of glucocorticoid agonists and antagonists. Equilibrium constants derived from the rate constants for association and dissociation were in good agreement with those determined directly or by competition under equilibrium conditions. Kinetic parameters for the slowly dissociating form of binding detected by a non-equilibrium dextran/charcoal competitive binding assay reflected the nature and extent of functional-group substitution on the steroid nucleus, but bore no relation to the classification of steroids as glucocorticoid agonists or antagonists. It is concluded that the binding of antagonists that is detected by such methods is agonist-like binding, which is not relevant to their antiglucocorticoid actions. Both agonists and antagonists displayed Michaelis–Menten association kinetics, but this behaviour was much more pronounced for antagonists. This is attributed to the existence of a second form of steroid–receptor complex, which escapes detection by the usual assay methods as a result of a high rate of dissociation and which is quantitatively antagonist-specific under equilibrium conditions. Direct evidence for the existence of two forms of antagonist–receptor complex was provided by results showing that the dissociation of the glucocorticoid antagonist progesterone from the receptor was biphasic.

Although binding to intracellular receptors appears to be an obligatory step in the mechanism of action of glucocorticoids (Munck & Leung, 1977), not all steroids that interact with glucocorticoid receptors generate biological effects. Partial or complete antagonism of the actions of active glucocorticoids by structurally related steroids such as progesterone or 11-deoxycortisol (cortexolone) has been demonstrated both *in vivo* (Desser-Weist & Desser, 1977; Duncan & Duncan, 1979) and *in*

Trivial and systematic names of steroids mentioned in the text: aldosterone, 11 β ,21-dihydroxy-3,20-dioxopregn-4-en-18-al 18,11-hemiacetal; corticosterone, 11 β ,21-dihydroxypregn-4-ene-3,20-dione; 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-11 β ,17,21-trihydroxy-16 α -methylpregna-1,4-diene-3,20-dione; prednisolone, 11 β ,17,21-trihydroxypregna-1,4-diene-3,20-dione; progesterone, pregn-4-ene-3,20-dione; triamcinolone acetonide, 9 α -fluoro-11 β ,16 α ,17,21-tetrahydroxypregna-1,4-diene-3,20-dione 16 α ,17-acetonide.

vitro (Makman *et al.*, 1967; Munck & Brinck-Johnsen, 1968; Samuels & Tomkins, 1970; Dausse *et al.*, 1977); such antagonists bind to glucocorticoid receptors and inhibit the binding of biologically active steroids (Munck & Brinck-Johnsen, 1968; Rousseau *et al.*, 1972; Rousseau & Schmit, 1977). Steroids that bind to glucocorticoid receptors have been comprehensively classified as pure agonists, partial agonists/antagonists or pure antagonists in terms of their effects on the induction of tyrosine aminotransferase activity in hepatoma tissue-culture (HTC) cells (Samuels & Tomkins, 1970; Rousseau *et al.*, 1972; Rousseau & Schmit, 1977).

The existence of the two extreme categories represented by pure agonists and pure antagonists implies that any model for glucocorticoid action must provide for at least two states, active and inactive (Munck & Leung, 1977; Bell & Jones, 1979), and so it is reasonable to suppose that agonists and antagonists give rise to functionally distinct forms or states of steroid–receptor complex. The

initial interaction of active glucocorticoids with their receptors in the soluble cytosol fraction of target cells is followed by a temperature- and ionic-strength-dependent activation or transformation process that results in the steroid-receptor complex exhibiting an increased affinity for binding sites in nuclear chromatin (for a review see Munck & Leung, 1977). Glucocorticoid antagonists have no or only limited ability to stimulate the cytosol depletion and nuclear accumulation of the receptor, even though they compete with biologically active steroids for binding to the non-activated cytosol form (Rousseau *et al.*, 1973; Wira & Munck, 1974; Turnell *et al.*, 1974; Kaiser *et al.*, 1979). These observations suggest that pure antagonists are incapable of promoting receptor activation and that partial antagonists may do so only to the extent that they are also partial agonists; thus they appear to localize the site of discrimination between agonists and antagonists at the level of steroid interactions with the cytosol receptor. If this is the case, the interactions of antagonists with the receptor may differ, qualitatively or quantitatively, from those of agonists. To investigate this possibility, and to gain further insights into the mechanisms involved in steroid-receptor interactions, we have studied the binding characteristics of a number of glucocorticoid agonists and antagonists, with the use of rat thymus cytosol as the source of the glucocorticoid receptor.

Experimental

Materials

[1,2-³H]Aldosterone (53 Ci/mmol), [1,2-³H]corticosterone (50 Ci/mmol), [1,2-³H]cortisol (41 Ci/mmol), 11-deoxy[1,2-³H]corticosterone (40 Ci/mmol), 11-deoxy[1,2-³H]cortisol (44 Ci/mmol), [1,2-³H]dexamethasone (25 Ci/mmol), 17-hydroxy-[7-³H]progesterone (11 Ci/mmol), [6,7-³H]prednisolone (46 Ci/mmol), [1,2-³H]progesterone (49 Ci/mmol) and [1,2,4-³H]triamcinolone acetonide (23 Ci/mmol) were obtained from Amersham International, Amersham, Bucks., U.K. Non-radioactive dexamethasone was supplied by Sigma Chemical Co. (Poole, Dorset, U.K.). Neutralized activated charcoal and dithiothreitol were also from Sigma; Dextran T-70 was from Pharmacia Fine Chemicals (London W.5, U.K.). All other reagents were supplied by 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 approx. 4 mg of protein/ml, was removed free of floating fat and used immediately. Molybdate was omitted from the homogenization buffer for certain experiments, as detailed in the Results section.

Steroid-binding studies

Radioactive steroids were added to cytosol as solutions in incubation buffer (TEDKM buffer unless otherwise specified). Non-radioactive steroids were prepared as stock solutions in ethanol, diluted with incubation buffer (19:1, v/v) and added 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 dextran/charcoal competitive binding assay (Jones & Bell, 1980). Portions of the reaction mixtures were incubated at 0°C with an equal 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, or in some experiments at 3000 *g* for 1.5 min, samples (0.5 ml) of the supernatant fraction were removed for measurement of radioactivity. The time of incubation with dextran/charcoal varied between 5 and 20 min for discontinuous binding assays, depending on the radioactive ligand used; in some experiments cytosol was exposed continuously to dextran/charcoal and sampled at intervals. 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 in the absence of 10 μ M-dexamethasone; the difference in the amounts of protein-bound steroid between the two incubations was taken as specific binding.

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) with a Nuclear-Chicago mark 2 liquid-scintillation spectrometer. The counting efficiency was 30–35%; corrections for quenching were made by the channels-ratio method with calibration curves for ³H.

Results

Receptor stability and steroid binding in the presence of molybdate or glycerol

Rat thymus cytosol preparations were as a routine prepared and incubated in the presence of 10 mM-molybdate at 0°C in order to inhibit inactivation of the free receptor and activation (transformation) of the steroid–receptor complex (Leach *et al.*, 1979; Sando *et al.*, 1979); this ensured that the primary process of steroid–receptor interaction was being studied as free as possible of the influence of other reactions. Results shown in Fig. 1 demonstrate the dramatic stabilizing effect of molybdate on steroid binding; the rate constant for the loss of steroid-binding capacity at 0°C was decreased from $6.5 \times 10^{-4} \text{ min}^{-1}$ in the absence of molybdate to $5.1 \times 10^{-5} \text{ min}^{-1}$ in the presence of 10 mM-molybdate. This low rate of receptor inactivation permitted saturation analysis of the binding of [1,2-³H]dexamethasone to the receptor to be performed after incubation at 0°C for 24 h, time (approx. 5 times the $t_{1/2}$ for dissociation of the steroid–receptor complex) sufficient for the system to reach equilibrium. The value of the equilibrium dissociation constant (K_d) for [1,2-³H]dexamethasone obtained under these conditions was in good agreement with the quotient of rate constants (Table

1). The concentration of receptor binding sites for [1,2-³H]dexamethasone was of the order of 1 pmol/mg of cytosol protein.

Although an apparent protective effect of glycerol on steroid binding by rat thymus cytosol has been reported (Schaumburg, 1972), the stability of the glucocorticoid receptor was unchanged in the presence of 20% (v/v) glycerol (Fig. 1). The concentration of steroid bound by the receptor was at all times higher in the presence of glycerol than in its absence, however. Since steroid binding in this experiment was determined with a subsaturating concentration of [1,2-³H]dexamethasone, the results suggested that the affinity of the receptor for [1,2-³H]dexamethasone was increased in the presence of glycerol. This conclusion was confirmed by determination of the equilibrium dissociation constant and the rate constants for the interaction; the results presented in Table 1 show that the affinity of the receptor for [1,2-³H]dexamethasone was 3–4-fold higher in the presence of 20% (v/v) glycerol than in its absence and that a decrease in the rate constant for dissociation was predominantly responsible for this effect.

Association kinetics at low steroid concentrations

The binding of a number of different steroids to the molybdate-stabilized glucocorticoid receptor in rat thymus cytosol at 0°C was determined at intervals up to 20 min after the addition of low concentrations (5–10 nM) of steroid, concentrations substantially below those required to produce saturation of the receptor at equilibrium. The concentration of receptor binding sites was determined simultaneously by saturation analysis with [1,2-³H]dexamethasone. Plots of

Fig. 1. Stability of the glucocorticoid receptor in rat thymus cytosol at 0°C

Cytosol was prepared in the absence of molybdate and was subsequently incubated at 0°C with no additions (●), with 10 mM-molybdate (▲) or with 20% (v/v) glycerol (○). Samples were withdrawn at the times shown for the measurement of steroid-binding capacity by incubation for 2 h at 0°C with 20 nM-[1,2-³H]dexamethasone in the presence and in the absence of 10 μ M-dexamethasone. For full experimental details see the text.

$$[1/([S]_0 - [R]_0)] \cdot \ln \{([S]_0 - [SR])/([R]_0 - [SR])\}$$

(where S, R and SR represent steroid, receptor and steroid–receptor complex respectively) versus time were linear, indicating that the interaction was apparently of second-order under the conditions used, for all the steroids investigated. Values for the apparent second-order rate constants for association derived from the data are given in Table 2. The rate constants were similar for almost all of the steroids tested, not only for steroids of widely differing affinity but also for steroids of differing biological activity classification. The low value observed for [1,2-³H]aldosterone may have been due to the presence of both hemiacetal and aldehyde forms, with only one form binding to the receptor.

Kinetics of dissociation

The dissociation of tritiated steroids from the glucocorticoid receptor was studied after incubation to equilibrium. Dissociation of the labelled steroid was initiated at 0°C by the addition of 10 μ M-

Table 1. *Binding parameters for the interaction of [1,2-³H]dexamethasone with the glucocorticoid receptor at 0°C in the presence of molybdate or glycerol*

K_d values were determined by saturation analysis after incubation of cytosol (prepared in the absence of molybdate) with a range of concentrations of [1,2-³H]dexamethasone at 0°C, for 24 h with 10 mM-molybdate present or for 18 h with 20% (v/v) glycerol. For full experimental details see the text. Rate constants were determined as described in the text. Results are expressed as mean values $\pm$ S.E.M. (no of experiments).

Parameter	Molybdate	Glycerol
K_d (M)	$4.02 \times 10^{-9} \pm 0.51 \times 10^{-9}$ (6)	$1.20 \times 10^{-9} \pm 0.12 \times 10^{-9}$ (11)
k_{-1} (min ⁻¹)	$2.19 \times 10^{-3} \pm 0.29 \times 10^{-3}$ (6)	$8.64 \times 10^{-4} \pm 1.42 \times 10^{-4}$ (5)
k_{+1} , 2nd-order (M ⁻¹ ·min ⁻¹)	$8.83 \times 10^5 \pm 0.68 \times 10^5$ (5)	1.21×10^6 (1)
k_{-1}/k_{+1} (M)	2.48×10^{-9}	7.14×10^{-10}

Table 2. *Rate constants for the interaction of agonists and antagonists with the glucocorticoid receptor at 0°C*
Apparent second-order association rate constants, k_{+1} , and first-order dissociation rate constants, k_{-1} , were determined as described in the text. The classification of steroids as pure agonists (Ag), partial agonists/antagonists (Ag/An) or pure antagonists (An) is that of Rousseau & Schmit (1977). The results shown are mean values from two or more separate experiments.

Steroid	Classification	k_{+1} (M ⁻¹ ·min ⁻¹)	k_{-1} (min ⁻¹)	k_{-1}/k_{+1} (nM)
[1,2,4- ³ H]Triamcinolone acetonide	Ag	1.58×10^6	4.91×10^{-4}	0.31
[1,2- ³ H]Dexamethasone	Ag	8.83×10^5	2.19×10^{-3}	2.48
[6,7- ³ H]Prednisolone	Ag	4.52×10^5	9.29×10^{-3}	20.5
[1,2- ³ H]Corticosterone	Ag	6.94×10^5	1.26×10^{-2}	18.2
11-Deoxy[1,2- ³ H]corticosterone	Ag/An	5.08×10^5	2.36×10^{-2}	46.5
[1,2- ³ H]Progesterone	An	6.42×10^5	2.37×10^{-2}	36.9
[1,2- ³ H]Aldosterone	Ag/An	1.40×10^5	9.02×10^{-3}	64.5
[1,2- ³ H]Cortisol	Ag	3.26×10^5	2.78×10^{-2}	85.3
11-Deoxy[1,2- ³ H]cortisol	Ag/An	—	0.17	—
17-Hydroxy[7- ³ H]progesterone	An	—	>0.15	—

dexamethasone; rates of dissociation obtained by incubation with excess non-radioactive dexamethasone were identical with those generated after removal of unbound steroid by chromatography on Sephadex G-25 (Jones & Bell, 1980). It is important to note that the practical difficulties inherent in the use of a discontinuous binding assay meant that sampling times were a minimum of 5 min apart and that no samples were taken within the first 5 min after initiation of dissociation.

Under these experimental conditions, dissociation from the receptor appeared to be a simple monophasic first-order process for all the steroids tested. Rate constants for dissociation differed markedly for different steroids (Table 2). These variations in the rate constant for dissociation largely accounted for variations in binding affinity; values for the equilibrium constants for different steroids derived from the rate constants were in reasonably good agreement with values obtained from competitive binding studies at equilibrium (Jones & Bell, 1980) (Fig. 2). Dissociation rate constants (and equilibrium constants) for different steroids correlated well with the nature and extent of substitution of the steroid nucleus by functional groups, but bore no obvious relationship to their activity classification as agonists, partial agonists or antagonists.

Association kinetics at high steroid concentrations

Observations suggesting that second-order kinetics for the association of glucocorticoid agonists with their receptor represent an approximation valid only at low concentrations have been reported (Kaine *et al.*, 1975; Pratt *et al.*, 1975). When the binding process was studied over a wider range of steroid concentrations it was observed that Michaelis–Menten kinetics applied to the interaction. To determine whether glucocorticoid antagonists displayed similar behaviour, and to verify the original observations, the initial rate of steroid binding was determined after incubation of receptor preparations with a wide range of steroid concentrations (5–100 nM) for 5 min at 0°C. Steroids investigated in this way included two pure agonists, [1,2,4-³H]triamcinolone acetonide and [1,2-³H]dexamethasone, a partial agonist, 11-deoxy-[1,2-³H]corticosterone, and a pure antagonist, [1,2-³H]progesterone. Fig. 3 shows the results of one such experiment; initial binding data for [1,2-³H]dexamethasone and [1,2-³H]progesterone are plotted in double-reciprocal form. Both steroids displayed Michaelis–Menten kinetics, but this behaviour was considerably more pronounced for [1,2-³H]progesterone. Kinetic parameters derived from similar plots for the four steroids studied are listed in Table

Fig. 2. Relationship between kinetic and equilibrium binding parameters for different steroids

Values of k_{-1}/k_{+1} at 0°C for the steroids listed in Table 2 are plotted as a function of their K_d values at 0°C. K_d values were derived from K_i values determined relative to dexamethasone by competition (Jones & Bell, 1980) and the K_d for dexamethasone (Table 1). The line of equivalence is shown.

Fig. 3. Kinetics of association of [1,2- ^3H]dexamethasone and [1,2- ^3H]progesterone at 0°C

The initial rate of association, v , of [1,2- ^3H]dexamethasone (●) and [1,2- ^3H]progesterone (○) with the receptor was determined after incubation of cytosol with a range of concentrations of these steroids for 5 min at 0°C. Initial rates were normalized by dividing by the receptor concentration, n (determined by saturation analysis with [1,2- ^3H]dexamethasone). For full experimental details see the text.

3, and show that all these steroids exhibited Michaelis–Menten kinetics. The K_m values for [1,2,4- ^3H]triamcinolone acetonide and [1,2- ^3H]dexamethasone were similar, at about 250 nM, but were considerably lower for 11-deoxy[1,2- ^3H]corticosterone and [1,2- ^3H]progesterone, at 67 and 48 nM respectively; values for V displayed a similar trend. These observations suggest that there are significant quantitative differences in the binding characteristics of agonists and antagonists. As expected, apparent second-order rate constants for association derived from these data were similar for all four steroids (Table 3).

Evidence for two binding states for [1,2- ^3H]progesterone

The complex association kinetics observed for both agonists and antagonists suggested the existence of a relatively weak intermediate form of steroid–receptor complex, a form that would have escaped detection by the usual disequilibrium methods of assay because of a rapid rate of dissociation. Calculations based on the observed kinetic parameters indicated that a significant proportion of antagonist–receptor complexes (33% in the case of [1,2- ^3H]progesterone) would exist in the intermediate state under equilibrium conditions, whereas agonist–receptor complexes would be present almost exclusively in the tight-binding state (Table 4). Further evidence in support of this conclusion was obtained from two experimental approaches, both derived from a consideration of the kinetic parameters. In the first, the specific binding, at equilibrium, of [1,2- ^3H]progesterone and [1,2- ^3H]dexamethasone to portions of the same cytosol preparation was determined simultaneously. Failure of the assay system to detect a putative intermediate form of steroid–receptor complex would be expected to result in the underestimation of the concentration of receptor binding sites with [1,2- ^3H]progesterone, but not with [1,2- ^3H]dexamethasone. Fig. 4 shows the data obtained, plotted according to the Scatchard (1949) method. Even after correction for dissociation occurring during the binding assay, the extent of binding of [1,2- ^3H]progesterone at saturation was only 76% of that of [1,2- ^3H]dexamethasone.

In the second approach, the dissociation of [1,2- ^3H]progesterone from the putative intermediate complex was detected directly. Cytosol preparations were incubated to equilibrium at 0°C with [1,2- ^3H]progesterone or [1,2- ^3H]dexamethasone, in the presence and in the absence of 10 μM -dexamethasone; dissociation was initiated by addition of a suspension of dextran/charcoal and allowed to proceed in the presence of dextran/charcoal. Samples were then withdrawn at intervals for brief centrifugation and the determination of protein-bound steroid. The

Table 3. *Michaelis–Menten association rate parameters*

The constants were derived from least-squares plots of binding data obtained in experiments similar to that shown in Fig. 3. For each experiment the maximum velocity, V , was normalized by dividing by the receptor concentration, n (determined by saturation analysis with [1,2-³H]dexamethasone). Apparent second-order association rate constants, k_{+1} , were obtained from V/nK_m . The results shown are mean values from duplicate experiments.

Steroid	K_m (nM)	V/n (min ⁻¹)	k_{+1} (M ⁻¹ ·min ⁻¹)
[1,2,4- ³ H]Triamcinolone acetonide	243	0.250	1.03×10^6
[1,2- ³ H]Dexamethasone	254	0.246	9.68×10^5
11-Deoxy[1,2- ³ H]corticosterone	76.4	0.067	8.77×10^5
[1,2- ³ H]Progesterone	47.8	0.048	1.00×10^6

Table 4. *Derivation of binding constants for a two-stage model*

Binding constants were derived from the data in Tables 2 and 3 for the model shown below, on the assumption of rapid equilibration of the intermediate state (S = steroid, R = receptor):

Steroid	K_1 (nM)	k_{+2} (min ⁻¹)	k_{-2} (min ⁻¹)	k_{-2}/k_{+2}
[1,2,4- ³ H]Triamcinolone acetonide	243	0.250	4.91×10^{-4}	1.96×10^{-3}
[1,2- ³ H]Dexamethasone	254	0.246	2.19×10^{-3}	8.89×10^{-3}
11-Deoxy[1,2- ³ H]corticosterone	76.4	0.067	2.36×10^{-2}	0.35
[1,2- ³ H]Progesterone	47.8	0.048	2.37×10^{-2}	0.49

Fig. 4. *Equilibrium binding of [1,2-³H]dexamethasone and [1,2-³H]progesterone assessed with a non-equilibrium dextran/charcoal assay*

results obtained are shown in Fig. 5; they demonstrate that the dissociation of [1,2-³H]progesterone from the glucocorticoid receptor could be resolved into two components, the faster of which was only apparent within the first 5 min after the initiation of dissociation. In contrast, the dissociation of [1,2-³H]dexamethasone under the same conditions was monophasic. For [1,2-³H]progesterone, extrapolation of the slower component of dissociation to zero time revealed that 69% of the steroid bound at zero time was in this more-slowly dissociating form. The rate constant for dissociation of the more-rapidly dissociating component was estimated to be 0.73 min⁻¹.

The specific binding of a range of concentrations of [1,2-³H]dexamethasone (●) or [1,2-³H]progesterone (○) to portions of the same cytosol preparation was determined simultaneously after incubation for 24 h at 0°C in the presence and in the absence of 10 μM-dexamethasone. For full experimental details see the text. Lines of best fit obtained by the method of least squares are shown; for clarity, values for the concentration of bound steroid are expressed relative to the concentration of [1,2-³H]dexamethasone bound at saturation.

Fig. 5. Dissociation of [1,2-³H]dexamethasone and [1,2-³H]progesterone from the receptor at 0°C in the presence of dextran/charcoal

Cytosol was incubated with 20 nM-[1,2-³H]dexamethasone (●) or 20 nM-[1,2-³H]progesterone (○), in the presence and in the absence of 10 μM-dexamethasone, for 4.5 h at 0°C, then dissociation was initiated by the addition of an equal volume of a dextran/charcoal suspension. Samples were removed and centrifuged at the times indicated for the determination of the concentration of specifically bound steroid. For full experimental details see the text. Lines of best fit were obtained by the method of least squares.

Discussion

It has been recognized for some time that the high spontaneous rate of inactivation of glucocorticoid receptor in crude cytosol preparations from various tissues must compromise the validity of estimates of kinetic and equilibrium binding constants made with these preparations (Bell & Munck, 1973; Kaine *et al.*, 1975; Pratt *et al.*, 1975; Aranyi, 1979). This problem has now largely been overcome as a result of the discovery of the remarkable receptor-stabilizing properties of molybdate (Nielsen *et al.*, 1977; Sando *et al.*, 1979). Molybdate may act by inhibiting a dephosphorylation process associated with receptor inactivation (Leach *et al.*, 1979; Sando *et al.*, 1979). The kinetic constants reported in the present paper, and the equilibrium binding constants reported previously (Jones & Bell, 1980), were all determined in the presence of molybdate; they are consistent with each other, and can therefore be regarded as reliable estimates of binding parameters. Glycerol has also been widely regarded as a stabilizing agent for steroid receptors and has been used for this purpose in numerous investigations, but it is clear from observations reported in the present paper that it has no direct stabilizing effect on glucocorticoid receptors. The effect of glycerol on the affinity of steroids for the receptor can account

for its apparent protective effect under certain experimental conditions (Schaumburg, 1972), and it may indeed afford some protection to the receptor indirectly, since ligand binding itself exerts a stabilizing influence (Bell & Munck, 1973). The predominant action of glycerol was to lower the rate of dissociation of steroid from the glucocorticoid receptor; a similar effect has also been demonstrated for the progesterone receptor (Hansen *et al.*, 1976).

Apparent second-order association rate constants and first-order dissociation rate constants for the interaction of a number of different steroids with the molybdate-stabilized glucocorticoid receptor were determined at 0°C by using a standard form of dextran/charcoal competitive binding assay. From the results obtained, it is clear that differences in equilibrium binding affinity for different steroids can largely be accounted for by variations in the dissociation rate constant; for the steroids tested (with the exception of aldosterone), dissociation rate constants covered a 57-fold range, whereas second-order association rate constants differed by less than 5-fold. Essentially identical conclusions have been reported previously from more limited studies with unstabilized receptor preparations, and have been interpreted as indicating that the functional groups that distinguish the different steroids do not contribute to the association process, but only come into play to retain the steroid in the binding site once association has occurred (Bell & Munck, 1973).

Although there was a clear relationship between dissociation rate constants (and equilibrium constants) for different steroids and the nature and extent of functional group substitution on the steroid nucleus, there was no correlation between values for these constants and the activity classification of steroids as glucocorticoid agonists, partial agonists or antagonists. From this we conclude that the receptor binding of pure or partial antagonists, as well as pure agonists, detected by the usual assay methods, is to one and the same site on the same state or conformation of the receptor. In other words, the binding of antagonists determined under these experimental conditions is agonist-like binding and is not relevant to their actions as antiglucocorticoids. However, evidence obtained from three separate experimental approaches does point to the existence of a distinct quantitatively antagonist-specific state of the steroid–receptor complex, a state that is not detected by the usual non-equilibrium assays because of its high rate of dissociation. The existence of a second state was suggested by Pratt and his associates (Kaine *et al.*, 1975; Pratt *et al.*, 1975), who showed that glucocorticoid agonists display Michaelis–Menten association kinetics, but the present findings demonstrate that this type of kinetic behaviour is even more pronounced for

Scheme 1. General two-state model for the interaction of steroids with the glucocorticoid receptor

In (a) the model is shown with K_d values for the pure agonist dexamethasone, and in (b) with values for the antagonist progesterone. The inactive state of the receptor and complex respectively is denoted by R^1 and SR^1 , and the active state by R^2 and SR^2 .

glucocorticoid antagonists such as progesterone. When the binding parameters for a simple two-state model were calculated on the assumption of rapid equilibration of the intermediate state, they indicated that more than 99% of triamcinolone acetone–receptor or dexamethasone–receptor complexes would be in the tight-binding state at equilibrium; the value obtained from direct equilibrium binding experiments was 76%. The demonstration of biphasic dissociation kinetics for progesterone provides direct evidence for two binding states and an estimate of 69% for the proportion of progesterone–receptor complexes in the more slowly dissociating form.

If the two forms of steroid–receptor complex are equated with biologically active and inactive states, the data can be accommodated by the general two-state model shown in Scheme 1. However, the experimental observations would then suggest that progesterone should behave as a partial agonist instead of a pure antagonist, since two-thirds of the progesterone–receptor complex would be in the active state. We believe that this apparent anomaly may reflect the experimental conditions used, since the binding studies were performed at 0°C whereas biological activity was assessed at 37°C. The steroid-binding characteristics of the glucocorticoid receptor are known to change dramatically with temperature (Wolff *et al.*, 1978; Jones *et al.*, 1979), and it is possible that the position of equilibrium between the two states of the steroid–receptor complex will also change with increasing temperatures so as to favour the inactive state for antagonists such as progesterone.

High concentrations of pure or partial antagonists, but not pure agonists, have been shown to accelerate the rate of dissociation of radiolabelled dexamethasone from the steroid–receptor complex

(Suthers *et al.*, 1976; Jones & Bell, 1980; Svec *et al.*, 1980). This effect has been attributed to the presence on the receptor of a secondary, low-affinity, binding site specific for glucocorticoid antagonists, the occupancy of which produces negatively co-operative interactions with the primary, agonist-binding, site (Jones & Bell, 1980). By invoking Occam's razor, it is tempting to conclude that this negatively co-operative site on the active agonist-binding state of the steroid–receptor complex corresponds to the presumed antagonist-specific site on the inactive state; this would imply that there are separate but interacting binding sites on the receptor for agonists and antagonists (Bell & Jones, 1979, 1982).

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