Journal of Steroid Biochemistry, Vol. 11, pp. 1129 to 1134
Pergamon Press Ltd 1979. Printed in Great Britain

CHARACTERISTICS OF GLUCOCORTICOID BINDING TO MOUSE LIVER CYTOSOL

MARIO E. MIRÁS and ROBERT W. HARRISON
Endocrinology Division, Vanderbilt University School of Medicine, Nashville, TN U.S.A.

(*Received* 23 *May* 1978)

SUMMARY

Mouse liver cytosol contains a protein which binds the synthetic glucocorticoid, triamcinolone acetonide. Binding was analyzed by the charcoal assay and sucrose density gradient techniques. Sucrose density gradient analysis under low ionic strength conditions revealed a 7.4*S* peak of binding activity. This binding was altered to 4*S* under high ionic strength conditions. Mouse serum did not bind triamcinolone acetonide. Cytosol binding was of high affinity ($K_D = 3.7 \times 10^{-9}$ M) and the concentrations of sites was 3×10^{-13} mol/mg protein. Competitive binding analysis revealed a glucocorticoid preference which was very similar to that of the AtT-20 mouse pituitary tumor cell line glucocorticoid receptor. The degradation of cytosol binding at 37°C was slowed by the presence of sodium molybdate. This effect was not due to a measurable change in the dissociation rate of the receptor or to the sodium component of the molybdate. These studies show that mouse liver cytosol contains a glucocorticoid receptor with properties very similar to those of the AtT-20 cell glucocorticoid receptor.

INTRODUCTION

Current evidence strongly suggests that glucocorticoids affect virtually all animal tissues [1, 2] and that these effects result from alterations in gene expression [3]. A variety of evidence suggests that a class of soluble intracellular proteins termed receptors are essential mediators of these effects [3, 4]. These proteins have, therefore, been subjected to intense scientific scrutiny. We have previously studied a glucocorticoid receptor found in the cytosol of the AtT-20 mouse pituitary tumor cell line and characterized its ability to bind both natural and synthetic glucocorticoids [16] as well as its ability to interact with nuclei [5]. However, it is not known what similarities might exist between the AtT-20 tumor cell receptor and the glucocorticoid receptor present in normal mouse tissues. Therefore, we have studied glucocorticoid binding to mouse liver cytosol and compared these findings to those obtained with AtT-20 cell cytosol to determine if the receptors present in these two systems are similar. In addition we have studied the mechanism by which the ion molybdate appears to stabilize the cytosol receptor.

Our studies indicate that the cytosol glucocorticoid receptor present in both the AtT-20 cell and the mouse liver are similar.

MATERIALS AND METHODS

Materials

[^{3}H] Triamcinolone acetonide* (33.7 Ci/ml) was obtained from New England Nuclear Corporation. Unlabeled steroids were obtained from Sigma Chemical Corporation and from Steraloids. Other chemicals, unless otherwise noted were obtained from Sigma Chemical Corporation. Immature female Swiss mice were obtained from Harlan Industries.

Methods

Preparation of cytosol. Fresh livers were obtained from mice killed by cervical dislocation and placed immediately in ice-cold Tris–saline (150 mM NaCl, 10 mM Tris–HCl, pH 7.5). All subsequent procedures were performed at 4°C unless otherwise noted. The tissue was blotted free of excess buffer, weighed and homogenized (0.2 g/ml) in TETG buffer (50 mM Tris–HCl, 1.5 mM EDTA, 12 mM thioglycerol, pH 7.5) using a Polytron PT-10 (Brinkmann Instruments) at a low setting. The crude homogenate was centrifuged at 12,000 *g* for 10 min. The layer of floating lipid was drawn off and the crude low-speed cytoplasmic material was decanted and centrifuged at 150,000 *g* for 1 h. Another small fat plug was removed by aspiration and the particulate-free supernatant decanted and used in all of the experiments to be described here.

Charcoal assay. The charcoal assay was performed

* The trivial names used are: 11β, 21-Dihydroxy-4-pregnene-3, 20-dione, corticosterone; 17, 11β, 21-dihydroxy-4-pregnene-3, 20-dione, cortisol; 9α fluoro-11β, 16α, 17, 21-tetrahydroxy-1,4-pregnadiene-3, 20-dione 16, 17-acetal with acetone, triamcinolone acetonide; 9α fluoro-11β, 17, 21-trihydroxy-16α-methyl-1,4-pregnadiene-3, 20-dione, dexamethasone; 4-pregnene-3, 20-dione, progesterone; 21-hydroxy-4-pregnene-3, 20-dione, desoxycorticosterone; 17, 21-dihydroxy-4-pregnen-3, 20-dione, cortexolone; 11β, 21-dihydroxy-3, 20-dioxo-4-pregnen-18-al(11→18)-Lactol, Aldosterone; 17β hydroxy-4-androsten-3-one, testosterone; 21-hydroxy-4-pregnene-3, 11, 20-trione, 11-dehydrocorticosterone; 17β-hydroxy-4-androsten-17α-methyl-3-one, 4-methyltestosterone; 11α, 17, 21-trihydroxy-4-pregnene-3, 20-dione, eiphydrocortosone; 1,3,4,(10)-estratrien-3, 17β-diol, 17β-estradiol.

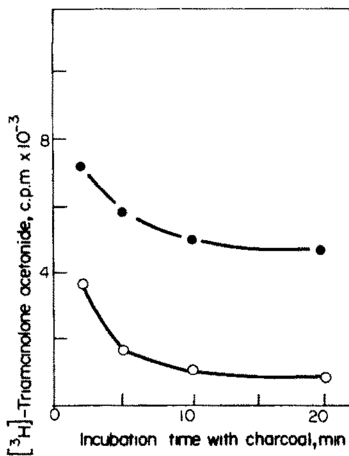

Fig. 1. Effect of length of charcoal incubation on binding. Cytosol was incubated at 4°C with labeled triamcinolone acetonide for 18 h. Duplicate incubations were done in the presence of a 1000-fold excess of unlabeled dexamethasone to estimate non-specific binding. Afterwards a 1% suspension of dextran-coated charcoal was added and incubated for 2, 5, 10 and 20 min. The closed circles represent total binding and the open circles represent non-displaceable or non-specific binding. The difference between the two lines represents specific binding.

essentially as described by Korenman[6]. A volume of Charcoal-Dextran solution (1% Norit-A (w/v), 0.2% Dextran-80 (w/v), 10 mM Tris, 1.5 mM EDTA, pH 7.5) equal to the volume of the experimental samples was added to cytosol containing radioactive steroid, incubated for various lengths of time and centrifuged at 2,000 *g*. The supernatant was decanted from the charcoal pellet and contained protein-bound steroid which was not adsorbed by the charcoal.

An important variable was the length of time required for adsorption of steroid. The experiment depicted in Fig. 1 showed that the reduction in non-specific binding (that which was not abolished by the addition of a 1000-fold excess of unlabeled dexamethasone) was maximal by 10 min. Total binding of labeled triamcinolone acetonide was also reduced after incubation with the charcoal-dextran solution for 10 min but the difference between the two lines, i.e. specific binding was maximal at this time point. Ten minutes was therefore chosen as the length of incubation in the charcoal assay. It was also established that the amount of the charcoal added was sufficient to adsorb all of the labeled steroid present even if there was no cytosol added.

Sucrose density gradient analysis. Sucrose density gradients (5–20% sucrose) were prepared in buffer containing 10 mM Tris–HCl 1.5 mM EDTA, 12 mM Thioglycerol and 10 or 300 mM KCl, pH 7.5, using a Beckman gradient-former. Labeled cytosol (0.3 ml) was layered on the gradient and centrifuged at 150,000 *g* for 16 h. The gradient profiles were analyzed after piercing the tube bottom, collecting 15-drop fractions and measuring the radioactivity in each fraction. Bovine Serum albumin (4.2*S*) was run in a separate gradient and the sedimentation coefficients of the radioactively-labeled proteins determined by reference to this standard [7].

Miscellaneous. Protein concentrations were determined as described by Lowry *et al.*[8]. Tritium was detected by counting 0.5 ml of an aqueous solution in 10 ml of ACS scintillation fluid (Amersham Searle) in a Beckman LS-200 scintillation counter. The counting efficiency for tritium was 40% as determined using an internal [^{3}H]-toluene standard.

RESULTS

Rate of association and dissociation

Following the addition of labeled triamcinolone acetonide to cytosol at 4°C binding increased rapidly to its maximal by 12 h and was stable for up to 72 h (Fig. 2). The addition of a 1000-fold (w/w) excess of unlabeled dexamethasone showed that binding was reversible with a dissociation half-time of 8 h*.

Sucrose density gradient analysis of triamcinolone acetonide binding

After centrifugation on low ionic strength gradients triamcinolone acetonide binding could be detected as a single peak with a sedimentation coefficient of 7.4*S*. This peak of binding activity was abolished by a 1000-fold excess of dexamethasone (Fig. 3). Non-glu-

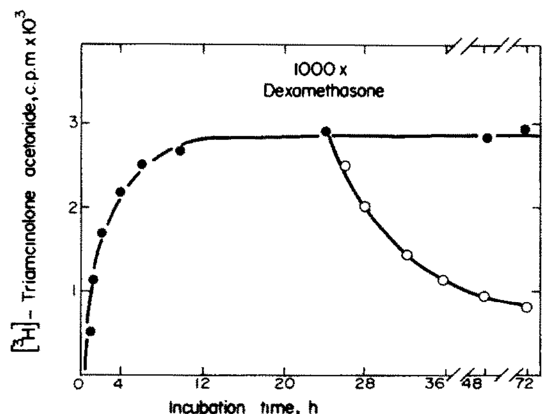

Fig. 2. Steroid association to and dissociation from the mouse liver cytosol receptor. Cytosol was incubated with 10^{-8} M labeled triamcinolone acetonide. A duplicate cytosol sample also contained unlabeled dexamethasone in order to estimate non-specific binding. At the indicated times aliquots (0.5 ml) of this cytosol were assayed by incubation for 10 min with an equal volume of a 1% suspension of dextran-coated charcoal (see Methods). At 24 h a 1000-fold excess of dexamethasone was added to portions of the cytosol samples and aliquots assayed to determine binding.

* The dissociation rate constant was 0.00127 M^{-1}. The association rate constant was $7.08 \times 10^5\ M^{-1}\ M^{-1}$ (for a description of how these rates were obtained see ref. [14]). The equilibrium dissociation constant calculated from these data is 1.78×10^{-9} M—a value very similar to that obtained by Scatchard analysis of equilibrium binding (Fig. 4).

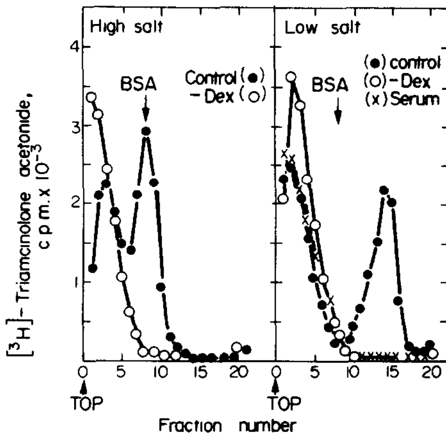

Fig. 3. Sucrose gradient analysis of mouse cytosol. Cytosol (0.3 ml), incubated for 2 h with 10^{-8} M labeled, triamcinolone acetonide, was layered on a 5–20% sucrose gradient and centrifuged at 150,000 g for 16 h and fractionated (for details see Methods). Cytosol incubated with labeled steroid alone (●) or with a 1000-fold excess of unlabeled dexamethasone (O). Serum (×) was diluted 1:20 with TETG and incubated with 10^{-8} M labeled triamcinolone acetonide.

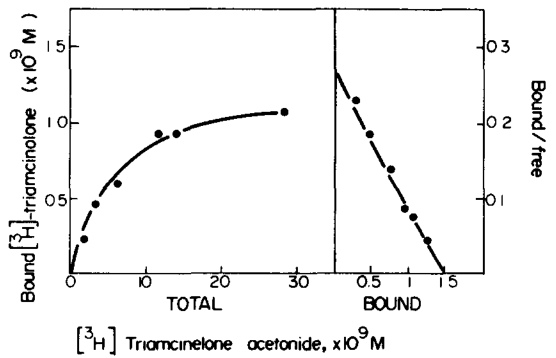

Fig. 4. Binding curve and Scatchard plot of cytosol binding. Cytosol was incubated for 18 h with 1–28 × 10^{-9} M labeled triamcinolone acetonide ± 1000-fold excess of unlabeled dexamethasone and assayed by the charcoal adsorption technique (see Methods). Cytosol protein concentration = 4.4 mg/ml. *Left Panel:* Specific binding at various steroid concentrations. *Right Panel:* Scatchard plot of specific binding.

cocorticoids such as estradiol-17β, aldosterone and testosterone did not effect binding but progesterone was an effective competitor (data not shown). Serum diluted 1:20 but otherwise labeled and analyzed in a fashion identical with cytosol did not demonstrate any binding activity (Fig. 3). Centrifugation through a gradient of high ionic strength resulted in the appearance of a slower sedimenting form with a coefficient of 4S. This binding was also abolished by the addition, to the cytosol, of a 1000-fold excess of dexamethasone simultaneously with the labeled triamcinolone acetonide.

Binding curve and Scatchard analysis

To determine the binding affinity of the receptor for its ligand and to estimate the concentration of receptor in cytosol the amount of specific binding was determined at several concentrations of labeled triamcinolone acetonide. Figure 4 shows a binding curve and Scatchard plot analysis [9] of triamcinolone acetonide binding to mouse liver cytosol. The binding curve (shown in the left panel) approached saturation at the highest concentration of steroid used. Scatchard analysis of the binding data (right panel) was linear indicating that the binding occurred to a single class of sites with a dissociation constant (K_D) of 3.7 × 10^{-9} M and that the binding site concentration was 3 × 10^{-13} mol/mg protein.

Binding specificity of the mouse liver receptor

The results of an experiment to test the ability of various steroids to compete with triamcinolone acetonide for binding sites are shown in Fig. 5. Specific binding of the labeled steroid to cytosol in the absence of unlabeled competitor is referred to as 100%. Unlabeled triamcinolone acetonide was the most effective competitor. Dexamethasone and progesterone were next in potency followed by corticosterone and cortisol. The *relative affinities** of each steroid were determined by dividing the molar concentration of the steroid required to produce 50% binding inhibition into the molar concentration of corticosterone required to inhibit binding by 50%.

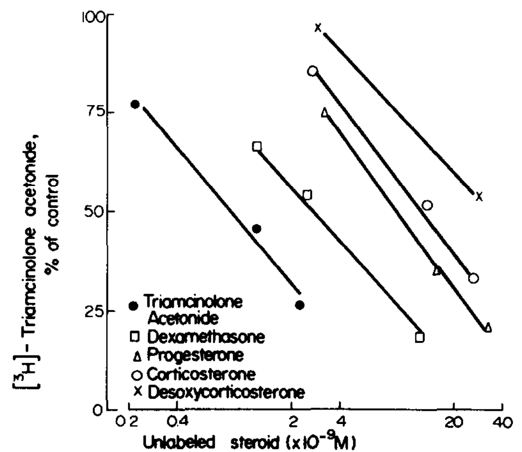

Fig. 5. Competitive binding analysis of binding to mouse liver cytosol. Cytosol was incubated with 10^{-9} M labeled triamcinolone acetonide and various concentrations of unlabeled steroids. Binding was determined by the charcoal assay technique. For details see Methods.

* These values have been used to quantitatively rank to ability of various steroids to compete with the labeled ligand for specific binding sites. These should not be construed as absolute values from which affinity constants can be calculated since the various criteria for that purpose have not been met (for a discussion of these criteria see ref. [15]).

Table 1. Relative binding affinities of various steroids for mouse liver and AtT-20 cell cytosol receptors

	Mouse liver	AtT-20 Cell
1. Triamcinolone Acetonide	17.20	10.1
2. Dexamethasone	4.95	3.9
3. Progesterone	1.82	2.38
4. Corticosterone	1.00	1.00
5. Desoxycorticosterone	0.38	0.67
6. Cortexolone	0.12	0.076
7. Cortisol	0.76	0.34
8. 11-Dihydrocorticosterone	0.02	0.007
9. 4-Methyltestosterone	0.01	0.005
10. 4-Androsten-11β-ol-3, 17-dione	0.003	0.001
11. Testosterone	0.008	0.004
12. Epihydrocortisone	0.001	<0.001
13. Estradiol-17β	0.000	0.000
14. Aldosterone	0.000	0.000

Competitive binding analysis was performed as described for Fig. 5. The relative binding affinity of a given steroid was determined by dividing the molar concentration of unlabeled steroid required to reduce binding to 50% by the concentration of unlabeled corticosterone required to produce the same reduction. The data for AtT-20 cell cytosol is taken from Harrison and Yeakley (in preparation).

Thus, a steroid with a higher binding affinity than corticosterone would have a *relative affinity* > 1. The results of these determinations show that the synthetic glucocorticoids triamcinolone acetonide and dexamethasone have the highest affinities (Table 1). Progesterone, an antiglucocorticoid, also had an affinity for the receptor which was greater than corticosterone. Aldosterone and estradiol-17β had no measurable affinity.

A purpose of this study was to determine if the glucocorticoid receptor in mouse liver cytosol was similar to the one found in AtT-20 cell cytosol. We reasoned that if the two proteins were similar their binding preferences would be similar. In Fig. 6 the binding preferences of each receptor are compared by plotting, for each steroid tested, the *relative affinity* obtained using AtT-20 cell cytosol *vs* the *relative affinity* obtained using mouse liver cytosol. The results, representing a 2000-fold range of values, shows that there is a linear relationship between the values obtained with either cytosol ($r = 0.99$ by the least square method).

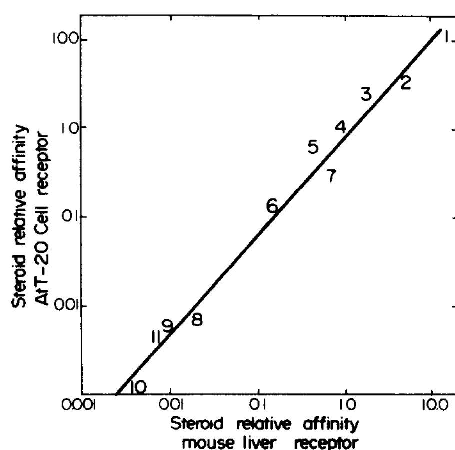

Fig. 6. Relationship of steroid relative affinities determined using AtT-20 cell cytosol and mouse liver cytosol. The relative affinities of various steroids were determined by comparing the molar concentrations needed to effect 50% displacement relative to corticosterone. The values were obtained for each steroid using mouse liver cytosol or AtT-20 cell cytosol. The numbers refer to the steroids listed in Table 1.

Effect of sodium molybdate on binding stability

It has recently been reported that sodium molybdate and other inorganic compounds can slow the degradation of glucocorticoid receptors in cytosol prepared from rat liver, rat thymocytes and mouse fibroblasts [10]. To determine if this was also true for this tissue, we tested the effect of sodium molyb-

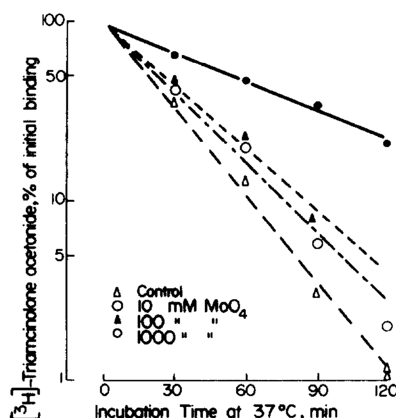

Fig. 7. Effect of sodium molybdate on receptor stability. Cytosol was labeled by incubation with 10^{-8} M triamcinolone acetonide at 4°C overnight. Various portions were then treated with sodium molybdate (10–1000 mM) and placed at 37°C. Binding at various times was determined by charcoal assay after cooling to 4°C. Control (△); 10 mM (○); 100 mM (▲); and 1000 mM (●).

date on the stability of triamcinolone acetonide binding at 37°C to mouse liver cytosol. The sodium molybdate concentrations employed in this experiment had no effect on the association rate of steroid with the receptor or on the eventual level of binding at equilibrium when tested at 4°C. The rate of dissociation of preformed steroid–receptor complex was also unaffected by 1 M sodium molybdate.

At 37°C approximately one-half of the steroid–receptor complex was degraded within 18 min. Sodium molybdate had a concentration-dependent protective effect on the receptor so that in the presence of 1 M sodium molybdate it took as long as 51 min for one-half of the binding to disappear. Similar concentrations of sodium ion, given as NaCl were not effective suggesting that the stabilization was due to the molybdate.

DISCUSSION

It is generally thought that steroid receptors for specific hormone classes may be similar in various species [11]. Although we have found the AtT-20 cell line ideal for many studies involving steroid–cell interactions, it is impractical for studies such as receptor purification which require large amounts of cytosol. Consequently, we have analyzed mouse liver cytosol to determine if it contains a glucocorticoid receptor and whether that receptor is similar to the one found in AtT-20 cell cytosol.

Triamcinolone acetonide was chosen as the labeled ligand because unlike corticosterone it was not affected by the large transcortin contamination from serum. Cytosol binding was reversible, of high affinity ($K_D = 3.7 \times 10^{-9}$ M) and to a limited number of sites (3×10^{-13} mol/mg protein). Since intact mice were used it is likely that the estimate of the binding site concentration was lowered by occupation of sites with endogenous hormone and by translocation of some sites into the nuclear compartment. The presence of a more slowly sedimenting form under high ionic strength conditions than under low ionic strength conditions on sucrose density gradient analysis is characteristic of cytosol steroid receptors [12].

The mouse liver receptor exhibited a clear glucocorticoid preference. In addition, a careful comparison of the binding specificity of the mouse liver receptor binding site to that of the AtT-20 cell cytosol receptor binding site suggests that they are similar. In contrast, we have found that the specificity of binding to the human placental glucocorticoid receptor is substantially different [17]. Furthermore, the binding affinity of this receptor for triamcinolone acetonide and its behavior on sucrose gradient analysis was very similar to that observed for the AtT-20 cell receptor [13]. These findings show that the mouse liver contains a cytosol glucocorticoid receptor and that it has characteristics which suggest that it is very similar to the glucocorticoid receptor found in cytosol of the AtT-20.

Our other findings confirm the observation by Nielsen *et al.*, that millimolar concentrations of molybdate ion partially protect the glucocorticoid receptor from degradation [10]. This effect was specific for the molybdate constituent and not due to simple changes in the receptor's binding kinetics.

Acknowledgements—We are grateful for the assistance of Ms. Katie Christian in the preparation of this manuscript. R.W.H. is an Investigator of the Howard Hughes Medical Institute. Supported by NCI Grant No. CA 19907 and the Vanderbilt University Diabetes-Endocrinology Research Center.

REFERENCES

1. Ballard P. L., Baxter J. D., Higgins S. J., Rousseau G. G. and Tomkins G. M.: General presence of glucocorticoid receptors in mammalian tissues. *Endocrinology* **94** (1974) 998–1002.
2. Leung K. and Munck A.: Peripheral actions of glucocorticoids. *Ann. Rev. Physiol.* **37** (1975) 245–272.
3. Baulieu E.-E., Atger M., Best-Belpomme M., Corvol P., Courvalin J. C., Mester J., Milgrom E., Robel P., Rochefort H. and DeCatalogne D.: Steroid hormone receptors. *Vitamins and Hormones* **33** (1975) 649–736.
4. Bourgeois S. and Newby R. F.: Diploid and haploid states of the glucocorticoid receptor gene of mouse lymphoid cell lines. *Cell* **11** (1977) 423–430.
5. Garroway N. W., Orth D. N. and Harrison R. W.: Binding of cytosol receptor-glucocorticoid complexes by isolated nuclei of glucocorticoid-responsive and nonresponsive cultured cells. *Endocrinology* **98** (1976) 1092–1100.
6. Korenman S. G.: Relation between estrogen inhibitory activity and binding to cytosol of rabbit and human uterus. *Endocrinology* **87** (1970) 1119–1123.
7. Martin R. G. and Ames B. N.: A method for determining the sedimentation behavior of enzymes. *J. biol. Chem.* **236** (1961) 1372–1379.
8. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193** (1951) 265–275.
9. Scatchard, G.: The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51** (1949) 660–672.
10. Nielsen C. J., Sando J. J., Vogel W. M. and Pratt W. B.: Glucocorticoid receptor inactivation under cell-free conditions. *J. biol. Chem.* **252** (1977) 7568–7578.
11. Greene G. L., Closs L. E., Fleming H., DeSombre E. R. and Jensen E. V.: Antibodies to estrogen receptor: Immunochemical similarity of estrophilin from various mammalian species. *Proc. natn. Acad. Sci. U.S.A.* **74** (1977) 3681–3685.
12. Stancel G. M., Leung K. M. T. and Gorski J.: Estrogen receptors in the rat uterus. Relationship between cytoplasmic and nuclear forms of the estrogen binding protein. *Biochemistry* **12** (1973) 2137–2141.
13. Watanabe H., Orth D. N. and Toft D. O.: Glucocorticoid receptors in pituitary tumor cells. *J. biol. Chem.* **248** (1973) 7625–7630.
14. Pratt W. B., Kaine J. L. and Pratt D. V.: The kinetics of glucocorticoid binding to the soluble specific binding protein of mouse fibroblasts. *J. biol. Chem.* **250** (1975) 4584–4591.
15. Rodbard D.: Mathematics of hormone-receptor interaction. In *Receptors for Reproductive Hormones* (Edited by B. W. O'Malley and A. R. Means). Plenum Press, New York (1973) pp. 289–326.

16. Harrison R. W. and Yeakley J.: Corticosterone binding in AtT-20 pituitary tumor cell cytosol *Biochim. Biophys Acta* **583** (1979) 360–369.

17. Speeg K. V. and Harrison R. W.: The ontogony of the human placental glucocorticoid receptor and inducibility of heat-stable alkaline phosphatase. *Endocrinology* **104** (1979) 1364–1368.