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# RECEPTOR TRANSLOCATION INHIBITOR AND APPARENT SATURABILITY OF THE NUCLEAR ACCEPTOR

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## SUMMARY

Steroid hormone–receptor complexes bind to nuclear acceptor(s). In various cell-free systems, this interaction has been considered as saturable, and this has led many research groups to postulate the existence of a relatively small population of acceptor sites with a high affinity for the complexes. Using rat liver nuclei, chromatin or DNA as acceptor, we have shown that at physiological concentrations of glucocorticoid receptor there is no actual saturation of the acceptor. A pseudo-saturation was observed, which was due to the inhibitory effect of non-receptor macromolecules present in the cytosol. Rather than a competition for the acceptor, the mechanism of this inhibition seems to consist of a direct interaction between the receptor and these unknown inhibitors. By repeated incubations with cytosol, it is possible to bind up to 21 pmol of $^3$H-dexamethasone–receptor complexes per mg DNA to the nuclei whereas the tissue concentration of receptor is 5·7 pmol/mg DNA. When rat liver slices are incubated at 37°C with $^3$H-dexamethasone, the equilibrium distribution of steroid–receptor complexes between nuclei and cytosol at all hormone concentrations is 9/1. It is therefore proposed that the interaction of steroid–receptor complexes with the nuclear acceptor is not a limiting factor in steroid hormone action since at all hormone concentrations a constant proportion of the complexes are bound to the nuclei.

The relevance of these findings to dose-response curves for steroid hormones is discussed. If it is accepted that the biological response is proportional to the amount of the ternary complexes (acceptor–receptor–steroid), then it may be calculated that the dose-response curve and the curve showing the degree of saturation of receptor at various hormone concentrations can be superimposed if one requirement is met. It is necessary that the cellular concentration of unbound steroid–receptor complexes never approaches the equilibrium dissociation constant of the interaction of these complexes with the acceptor sites implicated in the biological response. Two cases in which such a situation could possibly be present are described.

## INTRODUCTION

Steroid hormones are recognized in their target cells by specific receptors. Once it has bound the hormone, the receptor is translocated from the cytoplasm* to the nucleus (review in [1]). It is generally accepted that the information initially brought to the cell by the steroid molecule is secondarily carried inside the cell by the receptor–steroid complex in order to finally reach the chromatin where gene transcription is modified. At the present time a considerable amount of experimental work deals with the key problem of the characterization of the chromatin structure (called acceptor) involved in the formation of these ternary complexes (steroid–receptor–acceptor). The binding of steroid–receptor complexes to nuclear acceptor has been studied in cell-free systems derived from chick oviduct (progesterone) [2], rat uterus (estrogens) [3, 4], prostate (androgens) [5], liver [6] and hepatoma cells (glucocorticoids) [4, 7]. In all these cases the binding of various concentrations of receptor–steroid complexes to nuclei or chromatin was examined and the acceptor was found to be saturable. This led to the conclusion that steroid–receptor complexes are attached to a homogeneous set of acceptor sites. The equilibrium dissociation constant was in the $10^{-9}$–$10^{-10}$ M range (with the exception of [2]), and the number of acceptor sites was 500 to 15,000 per cell (lower than the intracellular concentration of receptor). The aim of the present work was to verify if such high affinity, low capacity, acceptors could be found in the case of rat liver nuclei and glucocorticoid receptors and to discuss the possible consequences of these characteristics of acceptors on dose-response relationships for steroid hormones.

* Actually there is no evidence that the receptor in the absence of the hormone is in the cytoplasm. It could well be loosely attached to a subcellular structure (endoplasmic reticulum, nucleus...) and be solubilized during the homogenization. Such a situation would not modify the following discussion.

† Methods were essentially the same as described in [8].

## RESULTS†

### I. *Apparent saturability of the binding of $^3$H-dexamethasone–receptor complexes to nuclei*

Rat liver nuclei were incubated with various concentrations of $^3$H-dexamethasone-saturated cytosol.

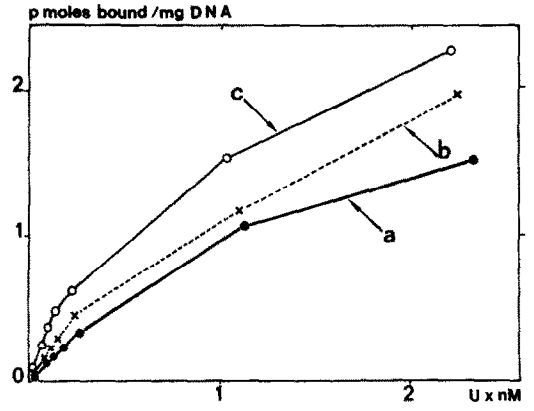


Fig. 1. Apparent saturability of the binding of $^3$H-dexamethasone–receptor complexes to rat liver nuclei.
Concentrated cytosol was incubated at 0° with a saturating concentration of $^3$H-dexamethasone.
(a) The incubate was activated (25°, 30 min), diluted in order to get various concentrations of steroid–receptor complexes, and incubated with nuclei (0°, 30 min).
(b) was similar to (a) except that the dilution was performed before the activation.
(c) the incubate was diluted and directly incubated with the nuclei (25°, 30 min).
U: concentration of soluble steroid–receptor complexes at the end of the incubation with the nuclei.

Receptor–steroid complexes bound to nuclei and remaining in the soluble fraction were measured. It may be seen (Fig. 1c) that the binding of $^3$H-dexamethasone–receptor complexes to the nuclei showed a pattern compatible with the presence of at least two populations of acceptor sites (one of high affinity and low capacity, and one of low affinity and high capacity). Scatchard analysis of these data (not shown) confirmed this interpretation. However, the binding of receptor–steroid complexes to the nuclei is actually a complex phenomenon which consists of at least two different steps: activation of receptor–steroid complexes and binding of the activated complexes to the nuclei. The activation may be defined as the appearance of a high affinity for nuclei. Activation takes place when the hormone becomes bound to the receptor but its rate is very slow at low temperature and low ionic strength. Thus in order to activate steroid–receptor complexes it is necessary to submit them temporarily to high temperature or high ionic strength. Once activated the complexes become able to bind to nuclei even at low temperature. Some evidence points to the possibility that the activation consists in the stabilization by the steroid of a conformation of the receptor having positive charges at its surface. The receptor–steroid complex then becomes able to bind to polyanions [8].

As in the experiment described in Fig. 1c, the incubation of the secondary complexes with the nuclei was performed at 25°C, two simultaneous phenomena took place: activation and binding to the nuclei. The observed saturation could not be clearly attributed to either of these. In order to solve this problem activation and binding were performed separately (Fig. 1a and 1b) with a similar result. The difference between curves a and b shows that activation is more effective in diluted cytosol and the difference between curves b and c demonstrates that either activation is more efficient in the presence of acceptor or that the binding to nuclei is also partially temperature dependent. When the same experiment was performed in 0·14 M KCl or using receptor-$^3$H-dexamethasone complexes which were partially purified by $(NH_4)_2SO_4$ precipitation and Sephadex G 200 chromatography, similar results were obtained [9]. In all cases there seemed to exist a saturation of at least one population of acceptor sites with increasing concentrations of secondary complexes [9].

II. *Evidence that the saturability of the acceptor is only apparent and does not correspond to a real saturation by dexamethasone–receptor complexes*

a. In the previous experiment the variation of the concentration of steroid–receptor complexes was obtained by the dilution of cytosol previously incubated with $^3$H-dexamethasone. However, in such a situation both the concentrations of receptor–steroid complexes and of non-receptor components present in the cytosol varied. To overcome this difficulty a fixed amount of cytosol was used in all the incubations with the nuclei but the concentration of $^3$H-dexamethasone was varied. Under these experimental conditions no saturation of the acceptor capacity of nuclei was noted in the presence of increasing concentrations of steroid–receptor complexes (Fig. 2). This experiment suggests that the pseudo-saturation observed in Fig. 1 is due to components other than receptor–steroid complexes present in the cytosol.

b. Steroid–receptor complexes but not free receptor bind to nuclei [8]. If the interaction of steroid–receptor complexes with the acceptor is truly saturable, the binding of $^3$H-dexamethasone–receptor complexes

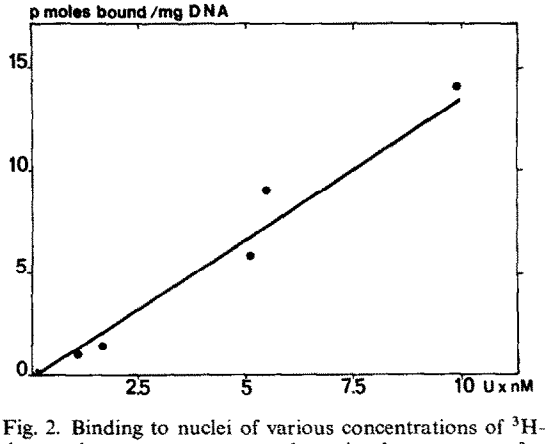


Fig. 2. Binding to nuclei of various concentrations of $^3$H-dexamethasone–receptor complexes in the presence of a fixed amount of cytosol.
Concentrated cytosol was incubated with various concentrations of $^3$H-dexamethasone (varying between 0·1 and 80 nM). Incubation with nuclei was then performed (25°, 30 min). U: Concentration of soluble steroid–receptor complexes at the end of the incubation with the nuclei.

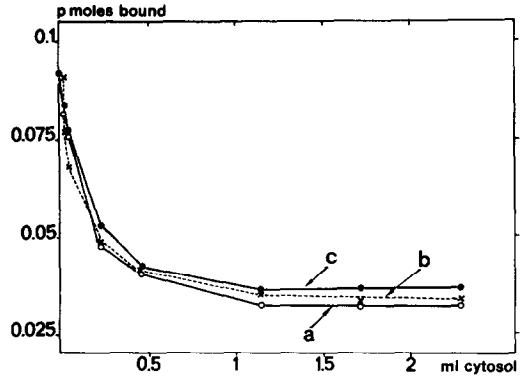


Fig. 3. Competition of the binding to nuclei of $^3$H-dexamethasone–receptor complexes by cytosol incubated with unlabelled dexamethasone (a), steroid-devoid cytosol (b) and receptor-devoid cytosol (c).

should be inhibited by complexes of unlabelled dexamethasone with receptor, but not by hormone-free receptor. Actually, as may be seen in Fig. 3, not only was steroid-devoid cytosol as effective as cytosol incubated with unlabelled dexamethasone in competing for $^3$H-dexamethasone–receptor binding, but even cytosol devoid of active receptor (prepared by heating at 37°C for 30 min in the absence of hormone) behaved identically.

c. If the concentration of acceptor sites is equal to or lower than the cellular concentration of receptor, and if the *in vitro* binding of receptor–steroid complexes takes place at the physiologically operating acceptor sites, then the following control experiment should be possible. The injection to adrenalectomized rats of a saturating dose of unlabelled dexamethasone provokes the transfer into the nuclei of most of the receptor (about 90%) complexed to hormone. The acceptor should then be saturated and when these nuclei are secondarily incubated *in vitro* with $^3$H-dexamethasone–receptor complexes, considerably diminished binding to the latter should be observed. Actually, no difference could be observed in the binding of $^3$H-dexamethasone–receptor complexes to control nuclei or to nuclei filled with unlabelled dexamethasone–receptor complexes (not shown).

d. A similar control can be performed *in vitro* by exposing the same nuclei to successive incubations in the presence of high concentrations of receptor–steroid complexes. As may be seen in Fig. 4, it is possible in 7 successive incubations to bind up to 21 pmol of $^3$H-dexamethasone–receptor complexes per mg of nuclear DNA. Some kind of limit is then attained. It is difficult to decide if this limit is due to a true saturation of the acceptor. Even if this corresponds to a saturation of the acceptor it does not correspond to the physiological situation (the cellular concentration of receptors is about 5·7 pmol/mg DNA).

III. *Pseudo-saturability of the binding of steroid–receptor complexes to chromatin and DNA*

When the concentration of steroid–receptor complexes was varied by dilution, the binding to chromatin and naked DNA appeared to be saturable. On the contrary when the concentration of the cytosol was kept constant and the concentration of receptor–steroid complexes was varied by addding different amounts of $^3$H-dexamethasone, the interaction appeared to be non-saturable [9].

IV. *Possible mechanisms of the pseudo-saturability of the nuclear acceptor*

Most of the evidence points to the fact that the apparent saturability of the formation of ternary complexes is actually due to the interference of non-receptor compounds present in the cytosol. Since they are precipitated by ammonium sulfate and recovered in the Sephadex G 200 void volume, they are probably macromolecules. Their inhibitory effect could take place at two levels: either in the nuclei (competition for the same site or alteration of the acceptor) or in the cytosol (interaction of the receptor with some inhibitory factors). The former mechanism can be easily explored by taking advantage of the fact that in the absence of the hormone, the receptor does not bind to the nuclei [8]. Nuclei were repeatedly incubated in the presence of hormone-devoid cytosol (at a concentration which inhibits steroid–receptor complex binding), and their ability to bind $^3$H-dexamethasone–receptor complexes examined. No difference was observed between treated and control nuclei. This demonstrated that the incubation with hormone-devoid cytosol did not lead to occupation of acceptor sites or to alteration of these sites [9]. Similar experiments showed the impossibility of removing inhibitory factors from cytosol by repeated

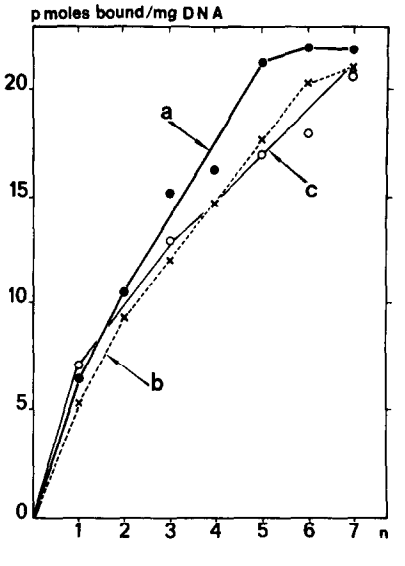


Fig. 4. Binding of $^3$H-dexamethasone–receptor complexes to nuclei on repeated incubations with $^3$H-dexamethasone-saturated cytosol.
Nuclei were incubated from 1 to 7 times at 0° with $^3$H-dexamethasone-saturated and activated cytosol.
a and b: represent two different experiments in which the incubations were performed at low ionic strength.
c: incubations were performed at 0·14 M KCl.
n: number of incubations of nuclei with cytosol.

incubation with nuclei [9]. These experiments, as well as the fact that the inhibitory effect is lowered by dilution or by a rise in ionic strength, point to the possibility that the inhibition is due to concentration-dependent receptor–macromolecule (possibly protein–protein) interactions in the cytosol.

V. *Distribution of $^3$H-dexamethasone–receptor complexes between nuclei and cytosol after incubation of rat liver slices*

Rat liver slices were incubated with various concentrations of $^3$H-dexamethasone at 37°C, until equilibrium was obtained. Receptor–steroid complexes were then measured in the nuclei and in the cytosol. It was found that over the entire range of steroid concentrations about 90% of the complexes were present in the nuclei (Fig. 5). If the concentration of acceptor sites was truly lower than that of the receptor, an opposite result would have been obtained, *i.e.* at high secondary complex (receptor–steroid) concentrations, the ratio of nuclear/cytoplasmic complexes should have been decreased.

## DISCUSSION

I. *Receptor translocation inhibitor*

While studying the interaction of $^3$H-dexamethasone–receptor complexes with rat liver nuclear acceptor, we have observed the existence of an apparent saturation of the acceptor. This finding was in agreement with previously published data on various systems [2–7]. However, various controls led us to conclude that there existed no real saturation, and that the observed pseudo-saturation was probably due to the presence in the cytosol of an inhibitor of receptor translocation. This inhibitor is a macromolecule (possibly protein) and interacts in a concentration-dependent way with the receptor–steroid complex. It would be of interest to study if this inhibitor is non-specific or if various specific inhibitors can be found for different receptors and for different cell types. It is also unknown if this inhibitor of steroid–receptor complex translocation from cytosol to nuclei, acts only in acellular conditions or if it also has an effect *in vivo*. For instance the fact that not all steroid–receptor complexes are bound by nuclei during tissue or cell incubations or *in vivo* administration of the hormone, could eventually be due to the presence of this inhibitor (partition of the activated complexes between the nuclei and the cytosoluble inhibitor).

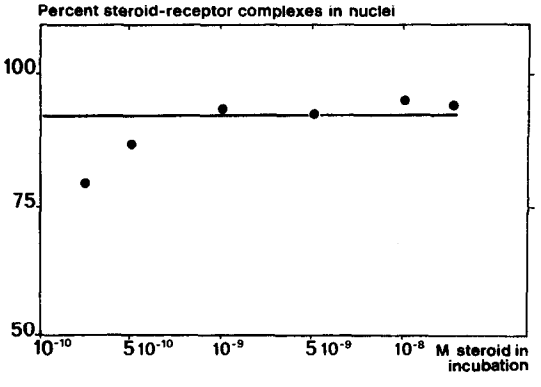


Fig. 5. Distribution between nuclei and cytosol of $^3$H-dexamethasone–receptor complexes after incubation of liver slices with various concentrations of $^3$H-dexamethasone. Liver slices were incubated (37°, 60 min) with various concentrations of $^3$H-dexamethasone (0·25–25 nM). The sum of the concentration of specific complexes in nuclei and cytosol is considered as the total concentration of steroid–receptor complexes at the end of the incubation.

II. *Non-saturability by receptor–steroid complexes of the nuclear acceptor*

During the completion of this work, a paper was published by Chamness *et al.*[10] which reported the absence of acceptor saturability in the case of estrogen receptors. A possible criticism of such experiments is that these cell-free systems do not reproduce the conditions existing in cells and that totally artefactual interactions may prevail which prevent observation of the true saturable acceptor. However, the results of binding studies performed under experimental conditions where the cellular structure was conserved, stand against this interpretation. On incubating liver slices with $^3$H-dexamethasone and studying the distribution of hormone–steroid complexes, we could not observe a saturation of the acceptor. At all hormone concentrations, a constant proportion of the total steroid–receptor complexes was present in the nuclei. A similar observation has been made by Williams and Gorski[11] when studying the binding of estrogen receptors to rat uterine nuclei. In another type of experiment, where the physiological situation was even more closely approached, De Hertogh *et al.*[12, 13] using *in vivo* perfusions of $^3$H-estradiol, found that in the entire range of hormonal concentrations, 75% of the estradiol was associated with the nuclei at steady state. In these experiments the association of the hormone with the uterus obeyed a simple hyperbolic law showing that only the binding to the specific protein (receptor) was observed.

It thus seems likely that in the target cells, the binding of steroid–receptor complexes to the acceptor is not a saturable phenomenon.

III. *Acceptor characteristics and dose-response curves*

Many possibilities appear when attempting to relate these findings to a possible mechanism of action of steroid hormones. It is a generally accepted working hypothesis that the biological response to a steroid hormone is directly related to the concentration of ternary complexes (steroid–receptor–acceptor) which are formed. In this respect the dose-response curve should be identical to the curve representing the degree of saturation of the acceptor at various steroid concentrations. The maximal response being obtained with the maximal occupancy of acceptor (concentration of ternary complexes when the receptor is saturated by steroid). If these hypotheses are accepted different mechanisms can be proposed.

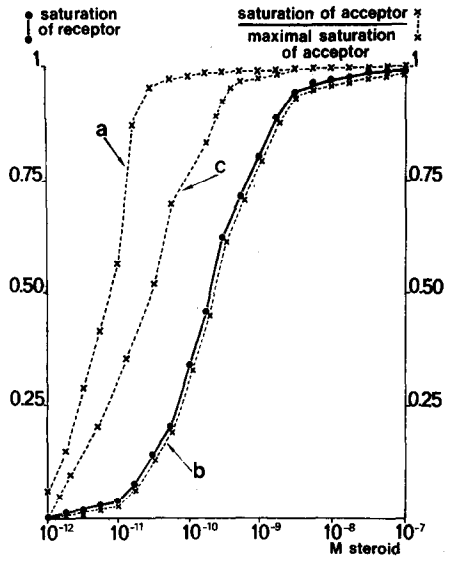


Fig. 6. Comparison in three model situations of the degree of saturation of the receptor with the degree of saturation of the acceptor at various concentrations of steroid.

The chosen characteristics of steroid–receptor interaction were in all cases: concentration of receptor binding sites 20 nM (concentration observed in the prepubertal rat uterus for estrogen receptor and in adrenalectomized rat liver for glucocorticoid receptor), $K_D = 0{\cdot}3$ nM (as observed for the interaction at 37° of estradiol with rat uterus receptor [20].

Three model situations were chosen for the interaction of acceptor with steroid–receptor complexes:

a: $K_D = 0{\cdot}1$ nM, concentration of acceptor sites 1 nM.

b: $K_D = 10$ nM, concentration of acceptor sites 100 nM.

c: simultaneous presence of both types of acceptor sites (as described in a and b). In this case the curve c describes the degree of saturation of the "specific" high affinity acceptor.

The degree of saturation of the acceptor (broken lines) at different steroid concentration is supposed to be identical to the dose-response curve (see text). All the calculations were made applying the law of mass action to the two successive equilibria [(1) steroid + receptor ⇄ steroid-receptor, (2) steroid-receptor + acceptor ⇄ steroid-receptor-acceptor]. The maximal saturation of acceptor is obtained when the receptor is saturated by steroid.

The first possibility would be the existence of a limited set of high affinity acceptor sites (similar to those described in 2–7) all implicated in the physiological response. In such a situation the effect of the hormone would be obtained at markedly lower concentrations than those necessary to obtain the binding of the steroid to the receptor. For instance, in the model situation described in Fig. 6a, 50% maximal binding to receptor would be observed at 0·3 nM steroid concentration, whereas 50% maximal response would be obtained at 9 pM. In this case, the acceptor is a limiting factor in steroid hormone action but we have already seen that evidence from this and other papers points against the occurrence of such a situation.

A second possibility could be the existence of a large concentration (higher than that of receptor) of acceptor sites all implicated in the physiological responses. In such a situation the acceptor would not be a limiting factor in hormonal action, since at all steroid concentrations a constant proportion of the steroid–receptor complexes would be bound to the acceptor. As shown in the model situation of Fig. 6b, the dose-response curve would be superimposed on the curve representing the binding of steroid to receptor.

A third possibility could be the presence in the nuclei of two populations of acceptor: high affinity, low capacity acceptor sites implicated in the physiological response ("specific" acceptors) and lower affinity, high capacity acceptor sites not implicated in the physiological response ("non-specific" acceptors). Here again the acceptor would be a limiting factor in hormone action since the physiological effect would depend not only on the degree of saturation of receptor by steroid but also on the degree of saturation of acceptor by secondary complexes. In Fig. 6c such a situation is shown. It may be seen that the dose-response and steroid binding to receptor curves are not superimposed. In this model 50% saturation of receptor is obtained at 0·3 nM steroid, 50% maximal response at 0·03 nM. However, a special situation would be obtained if the concentration or the affinity constant of the "non-specific" acceptor was high enough to decrease the concentration of secondary complexes to an extent where in the entire range of steroid concentrations it would be markedly lower than the $K_D$ of the interaction between the "specific" acceptor and steroid–receptor complexes. In this case, the acceptor would not be a limiting factor in hormone action since again the "specific" acceptor could not be saturated by the secondary complexes. Incidentally, it must be emphasized that the concentration of soluble secondary complexes as measured experimentally cannot be equated with that of free complexes in equilibrium with the acceptor(s) since on one hand a fraction of these soluble complexes may not be activated and on the other hand some complexes could also interact with the soluble translocation inhibitor discussed above. In both cases a fraction of the complexes could not be available for binding to the acceptor [9].

Unfortunately, in most systems it is impossible to observe physiological effects of steroid hormones *in vitro*, and in some systems where these effects could be studied, and have been compared to the binding of steroid to receptor, either the temperatures at which the two types of experiments were performed have been different or the precision of the dose-response measurements was insufficient. Rough data are consistent with a similarity between dose-response and steroid binding to the receptor curves. If these results prove to be true then, as previously discussed, this will indicate that in the cell the concentration of unbound secondary complexes never approaches the equilibrium dissociation constant ($K_D$) of the secondary complexes with the physiologically effective acceptor. This situation can be achieved either by the existence in the cell of a large number of physiologically efficient acceptor sites with a relatively low

affinity for the secondary complexes or by the presence of a small number of high affinity "specific" sites and a very large number of "non-specific" acceptor sites which would bind most of the steroid–receptor complexes. This would diminish the cellular concentration of unbound activated secondary complexes to extremely low values which would not approach (even at receptor saturation) the $K_D$ of the interaction between "specific" acceptor and secondary complexes.

## REFERENCES


1. *Advances in the Biosciences* (Edited by G. Raspé). Pergamon Press Vieweg, Oxford, Vol. 7 (1971).
2. O'Malley B. W., Schrader W. T. and Spelsberg T. C.: In *Advances in Experimental Medicine and Biology* (Edited by B. W. O'Malley and A. R. Means). Plenum Publishing Corporation, New York, Vol. 36 (1973) pp. 174–196.
3. King R. J. B. and Gordon J.: *Nature New Biol.* **240** (1972) 185–187.
4. Higgins S. J., Rousseau G. G., Baxter J. D. and Tomkins G. M.: *J. biol. Chem.* **248** (1973) 5873–5879.
5. Mainwaring W. I. P. and Peterken B. M.: *Biochem. J.* **125** (1971) 285–295.
6. Kalimi M., Beato M. and Feigelson P.: *Biochemistry* **12** (1973) 3365–3371.
7. Higgins S. J., Rousseau G. G., Baxter J. D. and Tomkins G. M.: *J. biol. Chem.* **248** (1973) 5866–5872.
8. Milgrom E., Atger M. and Baulieu E. E.: *Biochemistry* **12** (1973) 5198–5205.
9. Milgrom E. and Atger M.: (unpublished results).
10. Chamness G. C., Jennings A. W. and McGuire W. L.: *Biochemistry* **13** (1974) 327–331.
11. Williams D. and Gorski J.: *Acta endocr., Copenh.* suppl. **168** (1972) 420–438.
12. De Hertogh R., Ekka E., Vanderheyden I. and Hoet J. J.: *J. steroid Biochem.* **4** (1973) 289–299.
13. De Hertogh R., Ekka E., Vanderheyden I. and Hoet J. J.: *Endocrinology* **88** (1971) 165–174.
14. Munck A. and Wira C.: In *Advances in the Biosciences* (Edited by G. Raspé). Pergamon Press, Oxford, Vol. 7 (1971) pp. 301–330.
15. Baxter J. D. and Tomkins G. M.: In *Advances in the Biosciences* (Edited by G. Raspé). Pergamon Press, Vieweg, Oxford, Vol. 7 (1971) pp. 331–347.
16. Hackney J. F., Gross S. R., Aronow L. and Pratt W. B.: *Molec. Pharmac.* **6** (1970) 500–512.
17. Lippman M., Halterman R., Perry S., Leventhal B. and Thompson E. B.: *Nature New Biol.* **242** (1973) 157–158.
18. Katzenellenbogen B. S. and Gorski J.: *J. biol. Chem.* **247** (1972) 1229–1305.
19. Ruh T. S., Katzenellenbogen B. S., Katzenellenbogen J. A. and Gorski J.: *Endocrinology* **92** (1973) 125–134.
20. Milgrom E., Atger M. and Baulieu E. E.: *Biochim. biophys. Acta* **320** (1973) 267–283.