Acidophilic Activation of Steroid Hormone Receptors†

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ABSTRACT: In the absence of the hormone which they physiologically bind, steroid hormone receptors are present in the cytosol fraction of tissue homogenates. In the presence of the hormone, they become tightly bound to the nuclei. Using a cell-free system (cytosol or partially purified glucocorticoid receptor and nuclei from rat liver), it was shown that this transfer can be experimentally divided into three separate steps. The first step is the binding of the hormone by the receptor, which can take place at low temperature and low ionic strength. The second step is "activation" of the steroid receptor, defined as a rise in affinity for nuclei. The rate of the activation is highly dependent on temperature and ionic strength. Finally, the third step is the binding of the activated receptor–steroid complex to nuclei. This step, on the contrary, is inhibited by elevated ionic strength. Activation of the receptor by increase of temperature or ionic strength is only possible if it has previously bound the hormone. It can also be obtained at a rate similar to that observed with crude cytosol when using partially purified receptor, suggesting but not proving that the activation is due to a conformational change and not to an enzymatically provoked modification. The activation is markedly inhibited by Ca^{2+}, whereas other divalent cations have only a very small effect. The affinity of activated dexamethasone–receptor complexes is raised not only for liver nuclei, but also for various polyanions (homologous and heterologous DNA, RNA, and even carboxymethyl- and sulfopropyl-Sephadex). Therefore the receptor–steroid complex may be present under two different conformations which possibly differ by the presence (activated form) or the absence (inactivated form) of positively charged groups at the surface of the molecule, thus changing its affinity for polyanions. The receptor in the absence of the hormone can be only in the inactive form. Taking as a model the binding to DNA, it was shown that "activation" through a transitory increase in temperature or ionic strength is not confined to glucocorticoid receptor. Preliminary results indicate that it may be general for receptor–steroid complexes since it was also observed with the estrogen receptor from rat uterus (confirming results obtained by other authors), the progesterone receptor from guinea pig uterus, and the aldosterone receptor from rat kidney.

Specific intracellular binding proteins for all steroid hormones have been described in their respective target organs (Raspé, 1971). Called receptors, they are generally found in the cytosol (high-speed supernatant) after tissue homogenization, provided that the animal is deprived of the hormone which these proteins bind. After *in vivo* injection of the hormone, most of the receptor–steroid complexes are found in the nuclei and can only be extracted by buffer of high ionic strength. The same observation can be made after *in vitro* incubation of the tissue with the hormone, but only if the temperature has been raised to 20–37°, as shown early by Brecher *et al.* (1967), Jensen *et al.* (1968), and Gorski *et al.* (1968). Many studies indicate that the steroid–receptor complex interaction with chromatin may be due to DNA (King and Gordon, 1972; Toft, 1972; André and Rochefort, 1973), acidic protein(s) (Spelsberg *et al.*, 1972), or both, or even ribonucleoproteins (Liao *et al.*, 1973). This seems to be a key problem in studying the mechanism of action of steroid hormones, since many data point to the possibility that their action is initially mediated through a modification of transcription (Raspé, 1971).

Corticosteroid receptors have been described in thymus (Munck and Wira, 1971), hepatoma cells (Baxter and Tomkins, 1971), and liver (Beato and Feigelson, 1972; Koblinsky *et al.*, 1972). This work shows that liver glucocorticosteroid cytosol receptor, after it has bound the hormone, can undergo a temperature and ionic strength dependent change which enables it to bind to various polyanions. The hormone allows this "activation" to take place once it is bound to the receptor. The same result holds for estradiol, progesterone, and aldosterone and their respective receptors. The phenomenon of temperature-dependent activation and some of its features have already been reported in the case of the uterus estradiol (Brecher *et al.*, 1970; Jensen *et al.*, 1971, 1972; Gschwendt and Hamilton, 1972; De Sombre *et al.*, 1972) and hepatoma cell glucocorticosteroid (Baxter *et al.*, 1972) receptors.

Material and Methods

Buffer. In most experiments the buffer used was Tris (0.01 M)–$MgCl_2$ (3 mM)–β-mercaptoethanol (1 mM)–sucrose (0.25 M), pH 7.4.

Steroids. [1,2-^{3}H]Dexamethasone[1] (22 Ci/mmol) and [1,2-^{3}H]aldosterone (49 Ci/mmol) were obtained from the Radiochemical Centre (Amersham). [6,7-^{3}H]-17β-Estradiol (46.6 Ci/mmol) and [1,2-^{3}H]progesterone (50.3 Ci/mmol) were from New England Nuclear.

Unlabeled chromatographically pure steroids were gifts of Roussel-UCLAF.

Polynucleotides. Calf thymus DNA, 23S ribosomal RNA from *Escherichia coli*, and tRNA from yeast were obtained from Miles Laboratories. *Clostridium perfringens* DNA was from Worthington Biochemical Corp.

Animals. Wistar rats (200–270 g) were adrenalectomized 4–8 days before the experiments. They drank water containing 0.9% NaCl.

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[1] 9-Fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione.

Preparation of Subcellular Fractions. The animals were killed by a blow on the neck and decapitated. The liver was perfused with ice-cold saline and washed with buffer. All subsequent operations were performed at 0°. The tissue was usually homogenized in 4 vol of buffer in a glass–Teflon Potter-Elvehjem apparatus. Pure nuclei were prepared by the method of Chauveau *et al.* (1956) and resuspended usually in 5 ml of liver. The cytosol was prepared by a 105,000*g* (90 min) centrifugation.

Cytosol Incubation with the Steroid. [^{3}H]Dexamethasone in benzene containing 10% ethanol was introduced into a glass Packard counting vial. The solvent was evaporated under air and the vial kept at 0°. The cytosol was added and incubated with agitation for 2 hr at 0° time necessary to attain equilibrium. Parallel incubations were performed in the presence of a 170-fold excess of unlabeled hormone. The nonsaturable nonspecific binding was thus measured and subtracted from total binding in order to evaluate the specific saturable binding. All measurements in the cytosol and in the nuclei were corrected through this method, except when partially purified receptor was used, since in this instance the nonspecific binding was very low.

Partial purification of receptor was performed at 0°. Solid ammonium sulfate was added under stirring to the cytosol previously incubated with hormone until 30% saturation was obtained. After 30–60 min the precipitate was obtained by a 105,000*g* (15 min) centrifugation. This gave a tenfold purification with 80% recovery. When the receptor was used after this step, it was desalted by a passage through Sephadex G-25.

Occasionally it was further purified by chromatography on Sephadex G-200 (40 × 2.5 cm column). It was then recovered in the void volume giving an additional purification of about fourfold. When comparing with the initial cytosol there was a 40-fold purification with a 30% recovery.

Transfer of Receptor from Cytosol to Nuclei. Cytosol or partially purified receptor (0.5 ml) was incubated under agitation with 0.5 ml of a suspension of nuclei (300–600 μg of DNA). After the incubation the supernatant of a 3000*g* (10 min) centrifugation was used for the measurement of receptor-bound radioactivity. The pellet was washed twice with 3 ml of ice-cold buffer. Finally it was either directly counted for radioactivity or extracted for 20 min at 0° with 1 ml of buffer containing 0.4 M KCl, the supernatant of a 105,000*g* (10 min) centrifugation being called the nuclear extract.

In all the experiments it was verified that the binding of the receptor–steroid complexes to the nuclei was paralleled by a depletion of the complexes in the cytosol.

Assay of Receptor–Steroid Complexes in the Cytosol and in the Nuclear Extract. An ice-cold dextran (1 vol) (0.05%, w/v)–coated charcoal (0.5%, w/v) suspension in buffer devoid of sucrose was added to the solution and mixed with a Vortex for 2 sec. After 10-min incubation at 0° it was centrifuged at 3000*g* (5 min). The supernatant was counted for radioactivity. The dissociation of dexamethasone–receptor complexes is negligible within 10–15 min at 0°. In all cases a parallel solution incubated with a 170-fold excess of unlabeled dexamethasone was similarly processed in order to evaluate nonspecific binding.

DNA Preparation. DNA was prepared from rat liver using the technique of Stutz and Bernardi (1972) including as the last step chromatography on hydroxylapatite (Bernardi, 1971).

Radioactivity was counted either in a mixture of 10 ml of Omnifluor in toluene solution (4 g/l.) or in the Bray (1960) solution. External standardization was used for quenching correction. Proteins were assayed by the Lowry *et al.* (1951) technique using bovine serum albumin as the standard. DNA was assayed according to Burton (1956).

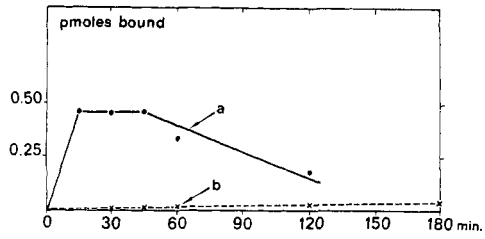

FIGURE 1: Temperature dependence of dexamethasone–receptor complex transfer into nuclei. The cytosol was incubated with [^{3}H]dexamethasone (6 nM) for 2 hr at 0°. Then cytosol and nuclei were incubated at 25° (a) and 0° (b) for various times. Radioactivity bound to nuclei was assayed after this last incubation. Correction has been made for nonspecific nonsaturable binding (see Materials and Methods).

Results

Conditions for the Transfer of the Cytosol Glucocorticoid Receptor to Nuclei in a Cell-Free System. When rat liver cytosol previously incubated with [^{3}H]dexamethasone at 0° was incubated at 25° with liver nuclei a part of the radioactive steroid became bound to them (Table I). About 80% of this radioactivity could be extracted from the nuclei with buffer of high ionic strength and 65% of it was macromolecule-bound steroid. The following evidence suggests that it was the receptor-bound radioactivity which was transferred. When the cytosol is incubated simultaneously with radioactive tracer and an excess of unlabeled hormone, the [^{3}H]dexamethasone is displaced from its saturable binding on the receptor and in a parallel manner the nuclear transfer of radioactive hormone is abolished. Treatment by *p*-hydroxymercuribenzoate, known to abolish hormone binding by the receptor (Koblinsky *et al.*, 1972), also suppresses the transfer. The precipitate obtained with 30% saturation ammonium sulfate and which contains the receptor also promotes the transfer.

When hormone-free cytosol is incubated with nuclei and the latter secondarily incubated with [^{3}H]dexamethasone, there is no radioactivity associated with the nuclei. However, since in the absence of hormone the receptor is unstable it had to be checked that the lack of nuclear binding of receptor in these experiments was not due to its complete inactivation. At the end of the incubation with nuclei the receptor was assayed in the soluble fraction. Its concentration was 35% of that of parallel experiments performed in presence of the hormone. This shows that variation in receptor stability cannot explain the complete lack of receptor binding to nuclei in the absence of hormone. Thus no receptor binds to nuclei in the absence of the hormone. The transfer of receptor–dexamethasone complexes is also highly temperature dependent (Figure 1) since after, for instance, 30-min incubation at 0°, the nuclear binding is only 3% of that obtained after the same incubation at 25°.

Activation of the Receptor. ACTIVATION BY HEATING OF RECEPTOR–STEROID COMPLEXES IN THE CYTOSOL. There appeared to be three possibilities to explain the thermal rise in nuclear binding of steroid–receptor complexes. Heating could either modify the properties of the cytosolic receptor or those of the nuclear "acceptor"[2] or could finally favor the interaction between receptor and acceptor. To test these different hy-

[2] The "acceptor" is defined as the structure(s) responsible for receptor–steroid complexes binding in the nuclei.

TABLE I: Conditions for the Transfer of Cytosoluble Glucocorticoid Receptor to Nuclei in a Cell-Free System.[a]

Conditions	N	[^{3}H]Dexamethasone in the Nuclear Pellet ($\times 10^{13}$ mol)	Nuclear Radioact. Extractible by 0.4 M KCl (%)	Macromol.-Bound Radioact. in 0.4 M KCl Extract (%)
1. Cytosol incubated with [^{3}H]dexamethasone (6 nM) prior to incubation with nuclei	6	4.53 ± 0.16	79.8 ± 3.1	63.3 ± 2.5
2. Cytosol incubated with [^{3}H]dexamethasone (6 nM) and unlabeled dexamethasone (1 μM) prior to incubation with nuclei	6	0.22 ± 0.00		
Specific binding 1 minus 2		4.31		
3. Buffer incubated with [^{3}H]dexamethasone (6 nM) prior to incubation with nuclei	3	0.23 ± 0.02		
4. Buffer incubated with [^{3}H]dexamethasone (6 nM) and unlabeled dexamethasone (1 μM) prior to incubation with nuclei	3	0.12 ± 0.00		
Specific binding 3 minus 4		0.11		
5. Receptor incubated with [^{3}H]dexamethasone (6 nM) and partially purified by ammonium sulfate precipitation prior to incubation with nuclei[b]	3	3.93 ± 0.06	71.5 ± 2.8	65.2 ± 3.0
6. Cytosol incubated with [^{3}H]dexamethasone (6 nM) and *p*-hydroxymercuribenzoate (1 mM) prior to incubation with nuclei	3	0.23 ± 0.00		
7. Cytosol directly incubated with nuclei; the nuclear pellet was secondarily incubated with [^{3}H]dexamethasone (6 nM)[c]	3	0.49 ± 0.06		
8. Cytosol directly incubated with nuclei; the nuclear pellet was secondarily incubated with [^{3}H]dexamethasone (6 nM) and unlabeled dexamethasone (1 μM)[c]	3	0.34 ± 0.05		
Specific binding 7 minus 8		0.15		

[a] Cytosol and nuclei were prepared as described under Materials and Methods. Cytosol was first incubated with hormone for 2 hr at 0° and then with nuclei for 30 min at 25°. The total concentration of receptor–steroid complexes in the cytosol was 4.7 nM. No correction has been made for nonspecific nonsaturable binding. N = number of incubations. Results are given as the mean ± the standard error of the mean. [b] The partially purified receptor solution contained 2.54 nM dexamethasone–receptor complexes. [c] In these experiments the cytosol was directly incubated with nuclei for 30 min at 25°. Nuclei were washed once and then incubated for 2 hr at 0° with hormone. The nuclear pellet was then washed twice.

potheses, cytosol previously incubated with steroid or nuclei was first heated separately at 25° and then incubated together at 0°. When the cytosol–dexamethasone incubate alone was preheated, the nuclear tranfer of complexes was raised (Figure 2A) to values similar to those observed when cytosol–nuclei incubations were performed at 25°. On the contrary, preheating of the nuclei did not modify the rate of transfer (not shown).

It thus appears that the increase by heating of receptor–steroid complexes binding to nuclei is related to a temperature-dependent change of the characteristics of the cytosolic receptor.

ACTIVATION BY HEATING OF RECEPTOR–STEROID COMPLEXES PARTIALLY PURIFIED BY AMMONIUM SULFATE PRECIPITATION. When receptor–steroid complexes partially purified by ammonium sulfate precipitation were used instead of crude cytosol, binding to the nuclei was observed even when the incubation was carried out at 0° (Figure 2B). This binding was, however, enhanced by preheating the partially purified complexes at 25°.

The question was then raised if exposure to ionic strength could replace heating in permitting receptor–dexamethasone complex activation.

ACTIVATION OF RECEPTOR–STEROID COMPLEXES BY EXPOSURE TO HIGH IONIC STRENGTH. Liver cytosol was incubated with [^{3}H]dexamethasone at 0° and at low ionic strength, and then the ionic strength was raised. After 2-hr exposure to various ionic strengths (0.1–0.6 M KCl) the incubate was diluted in order to bring back the ionic strength to 0.01 and it was incubated with nuclei at 0° for 30 min. The radioactivity bound to the nuclei was determined. Figure 3 shows that the temporary exposure to high ionic strength modifies the receptor–dexamethasone complexes in such a way that their affinity for nuclei is increased. This activation of receptor–steroid complexes by the increase of the ionic strength is the opposite of the effect of ions on the binding to nuclei of the already activated receptor. In the latter case (Figure 4) maximal binding is observed at the lower concentration of ions, whereas in 0.3–0.5 M KCl the binding is practically abolished. The rise of the ionic strength has thus completely opposite effects on the activation of dexamethasone–receptor complexes on the one hand and on the binding of activated complexes to nuclei on the other hand.

Effect of the Hormone on Receptor Activation by Heating or Increase of Ionic Strength. Liver cytosol was heated at 25° either after previous binding of hormone or in the absence of hormone. In the latter case hormone was secondarily added at

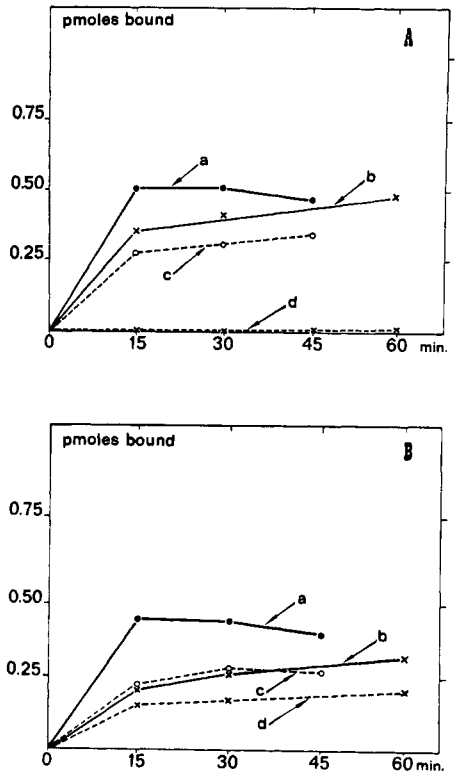

FIGURE 2: Activation of dexamethasone–receptor complexes by heating. (A) Activation of nonpurified dexamethasone–receptor complexes. Cytosol was incubated 2 hr at 0° with [^{3}H]dexamethasone (6 nM). Part of it was then preheated for 15 min at 25° and cooled for 15 min at 0°. Incubations with nuclei were carried out for various times either at 0 or 25°. Nuclear bound radioactivity was measured. Correction has been made for nonspecific nonsaturable binding (see Materials and Methods). (B) Activation of dexamethasone–receptor complexes partially purified by ammonium sulfate precipitation. Rat liver cytosol was incubated with 6 nM [^{3}H]dexamethasone for 2 hr at 0°. The hormone–receptor complexes were precipitated by ammonium sulfate (30% saturation) and desalted on Sephadex G-25 (see Materials and Methods). Aliquots of the purified preparations were preheated for 15 min at 25° and cooled for 15 min at 0°. Other aliquots were kept during this time at 0°. Incubations with the nuclei were then carried either at 0 or 25°. Nuclear-bound radioactivity was measured. The partially purified soluble preparation of receptor–dexamethasone complexes contained 0.36 nM [^{3}H]dexamethasone: (a) soluble fraction (cytosol or partially purified [^{3}H]dexamethasone–receptor complexes) preheated (25°) and incubated with nuclei at 25°; (b) soluble fraction preheated (25°) and incubated with nuclei at 0°; (c) soluble fraction kept at 0° and incubated with nuclei at 25°; (d) soluble fraction kept at 0° and incubated with nuclei at 0°.

0°. Transfer of receptor–steroid complexes to the nuclei was observed only if the activation by heating has been performed in the presence of hormone (Figure 5A). The rate of the thermal activation is relatively rapid; it is linear during the first 3–5 min and reaches maximal values after 5 min.

As the receptor is unstable in the absence of hormone, especially when the temperature is raised, the apparent absence of nuclear transfer of receptor in the absence of hormone could have eventually been related to the disappearance of the receptor. In order to test this hypothesis the concentration of dexamethasone–receptor complexes remaining in the soluble fraction at the end of the incubation with the nuclei was measured. Nuclear complexes could then be expressed as the percentage of the total (nuclei + soluble fraction) amount of recovered complexes at the end of the incubation. A curve very similar to Figure 5A was obtained, showing that the hormone

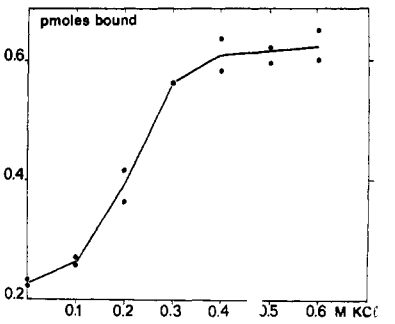

FIGURE 3: Activation of dexamethasone–receptor complexes by the rise of ionic strength. Liver cytosol, five times more concentrated than usual (homogenization in 0.8 volume), was prepared. It was incubated with 45 nM [^{3}H]dexamethasone for 2 hr at 0°. To aliquots of the incubate was added 0.25 vol of KCl buffer (KCl concentrations ranging from 3 to 0 M) giving final concentrations of KCl in the incubates varying from 0 to 0.6 M. After 2-hr exposure at 0°, 5 vol of buffer containing various concentrations of KCl was added in order to bring KCl concentrations in all the incubates to 0.01 M. The final concentration of [^{3}H]dexamethasone was 6 nM. Incubation with nuclei was then performed for 30 min at 0°. Nuclear-bound radioactivity was measured. Correction has been made for nonspecific nonsaturable binding (see Materials and Methods).

is truly necessary for the activation of the receptor (not shown). A similar experiment was performed but the activation was obtained through the rise of the ionic strength (Figure 5B). This rise was limited to 0.25 M KCl since at higher salt concentrations the receptor in the absence of hormone is very unstable. Here again it clearly appeared that the presence of the hormone was necessary during the activation process. It was also checked (not shown) that this effect could not be simply ascribed to stabilization of the receptor by the hormone.

The rate of the activation by exposure to 0.25 M KCl is very slow; it is linear up to the 60th min and is still increasing at 90 min. In the presence of the hormone, activation of the receptor is shown even at 0° and at a salt concentration of 0.01 M (Figure 6). The rate of activation in these experimental conditions was about 40-fold slower than with 0.25 M KCl at 0° and 1500-fold slower than in 0.01 M salt at 25°. Thus, the rises of temperature or ionic strength simply accelerate a change which, once the hormone is bound, takes place slowly even at low temperature and ionic strength.

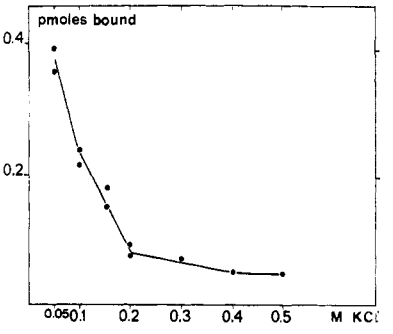

FIGURE 4: Effect of ionic strength on the binding to liver nuclei of [^{3}H]dexamethasone–receptor complexes previously activated. Cytosol was prepared and incubated with hormone as described in the caption to Figure 3. It was then activated for 2 hr at 0° by the addition of 0.25 vol of buffer containing 3 M KCl (final concentration in the incubate, 0.6 M). Finally 5 vol of buffer containing various concentrations of KCl (0–1.32 M) was added to aliquots and incubated for 30 min at 0° with 1 vol of nuclei. The final concentration of KCl varied between 0.05 and 0.5 M KCl. Nuclear-bound radioactivity was measured. Correction has been made for nonspecific nonsaturable binding (see Materials and Methods).

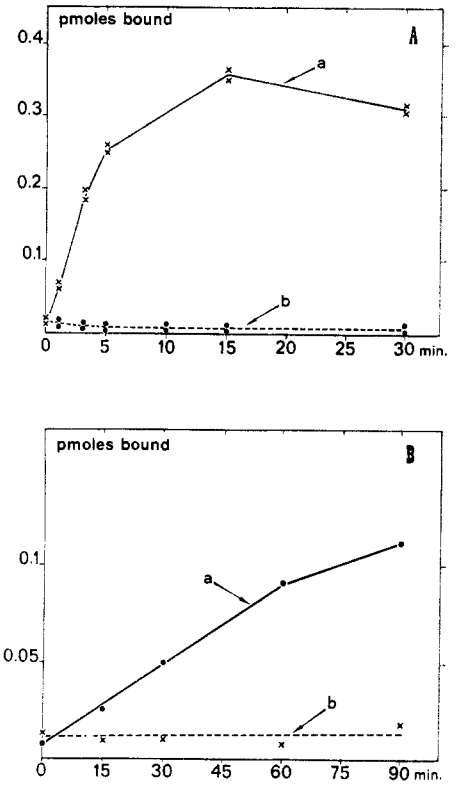

FIGURE 5: Activation of glucocorticoid receptor in the presence (a) or in the absence (b) of hormone. (A) Activation by heating. (a) Liver cytosol was prepared, incubated with 6 nM [^{3}H]dexamethasone for 2–2.5 hr at 0°, activated by heating at 25° for various periods of time, chilled for 15 min at 0°, and incubated in the presence of nuclei for 30 min at 0°. [^{3}H]Dexamethasone–receptor complexes bound to nuclei were measured at the end of the incubation. (b) Identical to experiment a, the only difference being that the incubation with 6 nM [^{3}H]dexamethasone (2 hr at 0°) followed the heating. (B) Activation by the increase of ionic strength. The experimental conditions were as described in the caption to Figure 3. (a) After binding of [^{3}H]dexamethasone for 2 hr at 0°, the receptor was activated by exposure for various times to 0.25 M KCl at 0°; then the cytosol was diluted and incubated with nuclei for 30 min at 0° in 0.02 M KCl. [^{3}H]Dexamethasone–receptor complexes bound to nuclei were measured at the end of the incubation. (b) Identical to experiment a, the only difference being that the incubation with 6 nM [^{3}H]dexamethasone (2 hr at 0° at 0.04 M KCl) succeeded to the exposure for various times to 0.25 M KCl. Correction has been made for nonspecific nonsaturable binding (see Materials and Methods).

Effect of Divalent Cations on Receptor Activation by Heating. Various divalent cations were added to the cytosol either prior to the activation step or after the activation but before the binding to nuclei (cytosol and nuclei were prepared in Mg^{2+}-free buffer). In the first case (Table II), only Ca^{2+} strongly inhibited the binding to the nuclei, whereas the effect of Mg^{2+} and Mn^{2+} was relatively small. The addition of EDTA to cytosol prepared in the absence of divalent cations was without effect. Ca^{2+} acted by inhibiting the activation step since when it was added after activation but prior to binding to nuclei its inhibitory effect was markedly diminished. Mg^{2+} and Mn^{2+} had a small inhibitory effect probably on the binding of the activated receptor to the nuclei, since addition of the ions prior to or after activation gave the same inhibition of the final binding to nuclei. The question may be raised of the relationship between this effect of Ca^{2+} and the receptor transforming factor described by Puca *et al.* (1972) for the estradiol receptor.

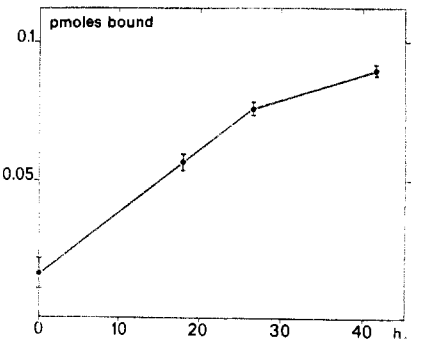

FIGURE 6: [^{3}H]Dexamethasone–receptor complex activation at 0° and at low salt concentration. Liver cytosol was incubated for 2 hr at 0° with 6 nM [^{3}H]dexamethasone. The incubate was then frozen in liquid nitrogen. After thawing it was allowed to stand for various times at 0° before incubation with nuclei for 30 min at 0°. [^{3}H]-Dexamethasone–receptor complexes bound to nuclei were measured (three measurements ± standard error of the mean). Correction has been made for nonspecific nonsaturable binding (see Materials and Methods).

Activation by the Increase of Temperature of Partially Purified Receptor. Activation of receptor–dexamethasone complexes can be due either to a conformational change of the receptor once it has bound the hormone or to a highly specific enzymatic action (the enzyme being active only on the hormone–receptor complex and not on the free receptor). In the latter case it should be possible to separate the enzyme from its substrate. In order to check this possibility the thermal activation of 40-fold purified receptor was examined. The rate of activation was similar to that observed with unfractionated cytosol (not shown).

Since the receptor was only partially purified, this experiment does not rule out the possibility that the activation is an enzymatic process. However, in this case the eventual enzyme should have coprecipitated with ammonium sulfate and cochromatographed on Sephadex G-200 with the receptor.

TABLE II: Effect of Divalent Ions on the Activation of Cytosoluble Receptor–[^{3}H]Dexamethasone Complexes and on Their Binding to Nuclei.[a]

	Ion Present during Activation and Incubation with Nuclei	Ion Present only during Incubation with Nuclei
No ion	100	100
Ca^{2+}	34.4 ± 0.6	83.7 ± 0.5
Mg^{2+}	74.7 ± 2.2	80.6 ± 2.7
Mn^{2+}	68.3 ± 1.3	81.3 ± 5.4

[a] Liver cytosol and nuclei were prepared in the absence of divalent ions. Cytosol was incubated with [^{3}H]dexamethasone (6 nM) at 0° for 2 hr. It was then activated for 15 min at 25° and cooled to 0° for 15 min. Cytosol was incubated with nuclei at 0° for 30 min. Nuclear-bound radioactivity was measured (it was 0.3 pmol in the control experiment). Divalent ions were added to a concentration of 3 mM either prior to the activation or only prior to the incubation with nuclei. Correction has been made for nonspecific nonsaturable binding (see Methods).

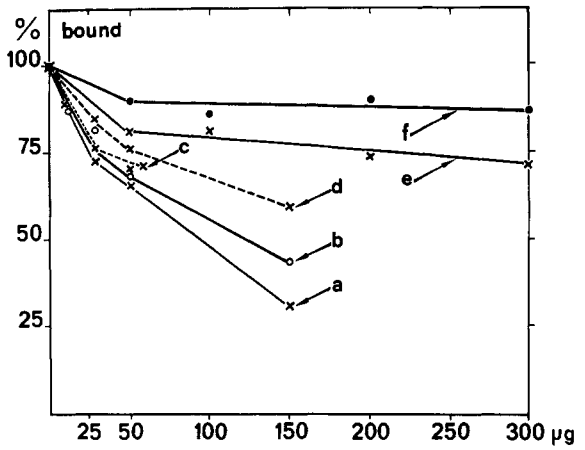

FIGURE 7: Competition between various polynucleotides and liver nuclei for activated [^{3}H]dexamethasone–receptor complexes. Rat liver cytosol two times more concentrated than usual (homogenization in 2 vol of buffer) was incubated with 12 nM [^{3}H]dexamethasone for 2 hr at 0° and then activated for 15 min at 25° and cooled for 15 min at 0°. Aliquots (0.25 ml) were incubated for 30 min at 0° with 0.25 ml of nuclear suspension (twice more concentrated than usual; resuspended in 2.5 ml of buffer/g of liver) and 0.5 ml of buffer containing various amounts of polynucleotides. Radioactivity bound to nuclei was measured. Correction has been made for nonspecific nonsaturable binding (see Materials and Methods): (a) rat liver DNA; (b) *Clostridium perfringens* DNA; (c) *E. coli* DNA; (d) calf thymus DNA; (e) *E. coli* 23S RNA; (f) tRNA from yeast.

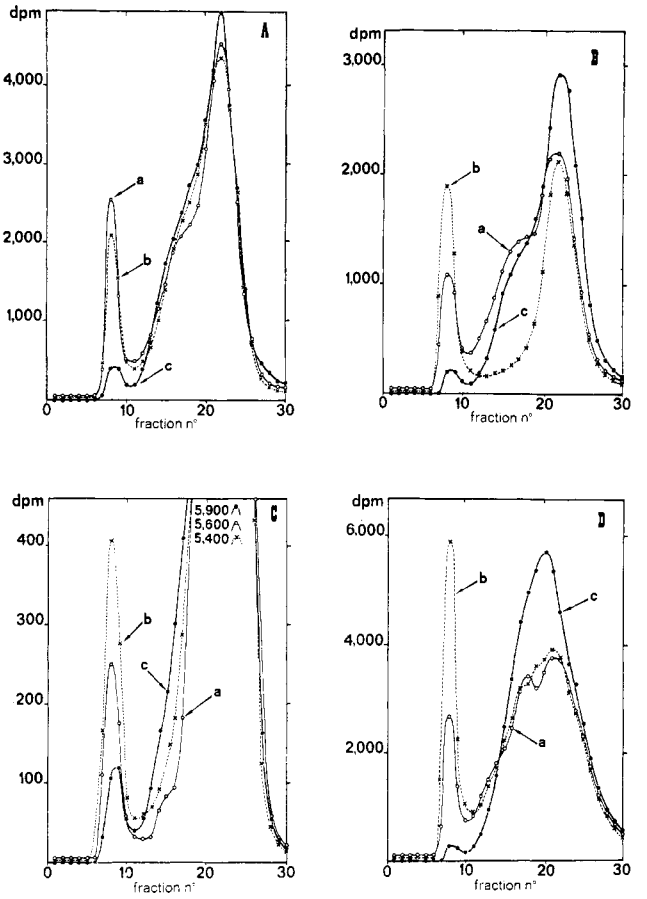

FIGURE 8: Binding of activated and nonactivated ^{3}H-labeled steroid–receptor complexes to DNA (rat liver glucocorticoid receptor (A), rat uterus estrogen receptor (B), rat kidney aldosterone receptor (C), and guinea pig uterus progesterone receptor (D)). Adrenalectomized adult male Wistar rats (A and C), 20–22-day-old female Wistar rats (B), and adult female Hartley guinea pigs (castrated 2 weeks previously and injected 3 days before the experiment with 5 µg of estradiol in 0.25 ml of sesame oil per day) (D) were used. Cytosols were five times more concentrated than usual (homogenization in 0.8 vol of buffer) and incubated for 2 hr at 0° with, respectively, 30 nM [^{3}H]dexamethasone and 14 nM [^{3}H]estradiol (sp act. 46.6 Ci/mmol), 14 nM [^{3}H]aldosterone (sp act. 49 Ci/mmol), and 42 nM [^{3}H]progesterone (sp act. 50.3 Ci/mmol). (a) Heat-activated complexes. To a part of the incubate was added 0.25 vol of buffer and it was heated for 15 min at 25°. After cooling for 15 min at 0°, it was diluted with 4 vol of buffer containing 0.1 M KCl (final concentration 0.08 M). It was then incubated with 1 vol of a solution of rat liver DNA (0.21 mg/ml); 0.4 ml was chromatographed on Sepharose 2B. (b) Ionic strength activated complexes. To a part of the incubate was added 0.25 vol of buffer containing 2 M KCl (final concentration 0.4 M). After 2 hr at 0° it was diluted with 4 vol of buffer (final concentration of KCl 0.08 M). It was processed as in a. (c) Nonactivated complexes. To a part of the incubate was added 0.25 vol of buffer. It was then diluted with 4 vol of buffer containing 0.1 M KCl and processed further as in a. Rat liver DNA was used with rat glucocorticoid, estrogen, and aldosterone receptors. Calf thymus DNA was used with guinea pig progesterone receptor.

With this purified preparation of receptor after 15-min activation at 25° the transfer of the complexes into the nuclei was 100%. Such a total transfer has never been observed with unfractionated cytosol suggesting that in this cytosol factor(s) exist inhibiting either the activation or the binding of receptor to nuclei.

"Acceptors" of Activated [^{3}H]Dexamethasone–Receptor Complexes. After exposure to heat or high ionic strength the affinity of [^{3}H]dexamethasone–receptor complexes for liver nuclei is increased. The question of the specificity of the liver nuclei as acceptors for activated receptor–steroid complexes was then raised.

Competition between Various Polynucleotides and Liver Nuclei for Activated [^{3}H]Dexamethasone–Receptor Complexes. Activated [^{3}H]dexamethasone–receptor complexes were incubated with either liver nuclei alone or mixtures of liver nuclei with various polynucleotides. Binding of complexes to nuclei was measured. It was observed (Figure 7) that all the tested polynucleotides had an inhibitory effect but that the inhibition due to various DNAs was more pronounced than the inhibition observed with yeast tRNA and *E. coli* 23S RNA. Rat liver DNA had apparently the higher affinity for the activated receptor.

The inhibition was probably underestimated in these experiments since 10–15% of the incubated DNAs stuck to the nuclei. Some of the complexes counted with the nuclei could have then been bound to this DNA and not to nuclei. There appeared to be no simple relationship between the affinity of the receptor for various DNAs and their base content.

Binding of Activated Receptor to DNA. Chromatography on Sepharose 2B can separate DNA from receptor–steroid complexes or unbound steroid (Baxter *et al.*, 1972). When activated [^{3}H]dexamethasone–*receptor* complexes are incubated with DNA and chromatographed, a peak of radioactivity appears with DNA in the exclusion volume. This binding to DNA necessitates the presence of the receptor; since it is not observed with free hormone, it can be abolished by the procedures which inactivate the receptor (heating to 60°, *p*-hydroxymercuribenzoate treatment) or which chase the radioactive steroid from it (incubation with an excess of unlabeled steroid) (M. Atger and E. Milgrom, manuscript in preparation). Figure 8A shows that the activation process can also be observed when the acceptor for [^{3}H]dexamethasone complexes is rat liver DNA. [^{3}H]Dexamethasone–*receptor* complexes kept at low temperature and salt concentration

TABLE III: Binding of Activated and Nonactivated [^{3}H]-Dexamethasone–Receptor Complexes to Liver Nuclei, Carboxymethyl-Sephadex, Sulfopropyl-Sephadex, and Glass Beads.[a]

	Bound [^{3}H]Dexamethasone–Receptor Complexes		
	Activated Complexes	Non-activated Complexes	Increase of Binding by Activation (%)
Liver nuclei	100[b]	9.2	1,090
Carboxymethyl-Sephadex	93.4	28.6	330
Sulfopropyl-Sephadex	37.7	11.8	320
Glass beads	47.8	14.1	340

[a] Liver cytosol was prepared and incubated for 2 hr at 0° with 6 nM [^{3}H]dexamethasone. A part of it was heated for 15 min at 25° and cooled for 15 min at 0°. Another part was kept at 0°. Aliquots (0.5 ml) of the cytosol were incubated for 30 min at 0° with 0.5 ml of a suspension containing either liver nuclei or carboxymethyl-Sephadex or sulfopropyl-Sephadex or glass beads. Particle-bound radioactivity was measured as usual except that three washes instead of two were performed. Correction has been made for nonspecific nonsaturable binding (see Materials and Methods). Carboxymethyl-Sephadex CM-50 and sulfopropyl-Sephadex SP-50 were thoroughly washed in the buffer until pH and ionic strength equilibrium and degassed before they were used. The suspension contained 11.8 mg (dry weight)/ml. Glass beads (ground glass diameter 63–100 μ) were washed five times with buffer. The suspension contained 200 mg/ml. [b] 0.25 pmol of activated [^{3}H]dexamethasone–receptor complexes were bound to liver nuclei.

show only a slight binding to DNA, whereas exposure to 25° or 0.4 M KCl raises markedly the affinity of the complexes for DNA.

The same phenomenon was observed with various heterologous DNAs from calf thymus or even bacteria (M. Atger and E. Milgrom, manuscript in preparation).

Binding of Activated Receptor to Carboxymethyl- and Sulfopropyl-Sephadex. Since activated dexamethasone–receptor complexes appeared to bind to all the tested polynucleotides, the question was asked if they were able to bind to such artificial polyanions as substituted dextrans; carboxymethyl- and sulfopropyl-Sephadex were incubated in the presence of either nonactivated or activated (by heating at 25°) [^{3}H]dexamethasone–receptor complexes. Table III shows that the activation resulted in a marked rise of affinity for the substituted dextrans. Binding of receptor to glass beads has been previously reported (Clark and Gorski, 1969). Using this "acceptor" the effect of activation could also be shown.

Activation of Estrogen, Mineralocorticoid, and Progestagen Receptors. In order to test if the activation was restricted to glucocorticoid receptors, or if it was a general phenomenon, estradiol, aldosterone, and progesterone receptors were incubated with their respective tritiated hormones. Aliquots of the incubate were either kept at 0° at low salt concentration, heated for 15 min at 25° and then cooled back to 0°, or submitted to 0.4 M KCl during 2 hr and then diluted back to low ionic strength. They were then incubated at 0° with DNA and chromatographed on Sepharose. It may be seen (Figure 8B–D) that for all the tested hormones previous exposure of steroid–receptor complexes to 25° or to 0.4 M KCl resulted in a several-fold increase of the affinity for DNA.

Discussion

Receptor–steroid complexes activated by a transitory rise in temperature or ionic strength become able to bind at low ionic strength and low temperature to a variety of natural or synthetic polyanions. It may thus be proposed as a working hypothesis that the activated receptor differs from the nonactivated receptor by the existence of positively charged regions at its surface. That the interaction between activated receptor and nuclei or DNA may involve ionic interactions is further shown by its disruption at higher ionic strength. According to this hypothesis the nuclear translocation of steroid receptors under the effect of the hormone can be summarized by the following sequence of events.

$$R_c \xrightarrow{+H} R_cH \longrightarrow R_c^+H \longrightarrow R_n^+H$$

In the absence of the hormone the receptor is found in the cytosol (R_c); once it has bound the hormone (H) the complex undergoes a transformation such that positive charge(s) appear at its surface (R_c^+H) and the activated cytosolic complex binds then to polyanionic "acceptor" in the nucleus (R_n^+H). The definition of the cytosolic receptor is purely operational (found in the 105,000g supernatant of the homogenate) since no evidence is available that in the absence of the hormone the receptor is located in the cytoplasm of the intact cell.

The change of ionic charges at the surface of the receptor during activation remains hypothetical and of unknown mechanism. Whether or not temperature or increased ionic strength provokes the same modification is also unknown. It is improbable that an enzyme is involved in the receptor activation since partial purification does not change the rate of the phenomenon. It is thus possible that activation is due to a conformational change of the receptor molecule. Most of these questions will be answered only with homogeneous receptor preparations. According to heat activation experiments, it has been proposed (De Sombre *et al.*, 1972) that the activation of the estrogen receptor is indicated by a shift of its sedimentation coefficient from 4 to 5 S, in sucrose gradients containing 0.3 M KCl. The present work shows that this latter ionic strength (established throughout the centrifugation period) activates even the previously nonheated receptor; however, the sedimentation coefficient is 4 S in these conditions. It may thus be premature to equate the change of sedimentation coefficient and the activation phenomenon. The same question has to be asked about the shift of the isoelectric point observed by Mainwaring and Irving (1973) after heating of the dihydrotestosterone–receptor complexes. In their case too, even the nonheated complex was probably activated by the prolonged exposure to high ionic strength during the isoelectric focusing. The activating effect of ionic strength is difficult to explain. A possible hypothesis could be that the postulated positive charges which appear on the surface of the activated receptor are engaged in some ionic interactions inside the nonactivated receptor. The binding of the hormone stabilizes a conformation of the receptor where these interactions must be suppressed in order to expose the positive charges. A rise of ionic strength could facilitate the rupture of these ionic interactions. The problem of the physiological "acceptor" of receptor–

steroid complexes in the nuclei is still unresolved. The finding that activated receptor can bind to various polyanions should incline to be very cautious when interactions between receptor and various structures are demonstrated *in vitro* and are then supposed to be the physiologically operating ones.

Acknowledgments

We thank Brigitte Leclaire for technical help.

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