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INFLUENCE OF TRIAMCINOLONE, ESTRADIOL-17β AND TESTOSTERONE ON 1,25-DIHYDROXYVITAMIN D_3 BINDING PERFORMANCES TO ITS CHICK INTESTINAL RECEPTOR

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Summary—We have investigated the effects of large molar excesses (20,000) of triamcinolone, estradiol-17β and testosterone on the binding performances of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] to its receptor. The source of receptor was a duodenal chromatin fraction of vitamin D replete chickens which exhibited a high level of positive cooperativity (Hill coefficient = 1.50 ± 0.12; $n = 4$) in the binding of 1,25$(OH)_2D_3$ to the two sites of its intestinal receptor. Triamcinolone did not modify the affinity, cooperativity level and maximum binding capacity of the 1,25$(OH)_2D_3$ receptor. Estradiol-17β induced a slight but significant increase of $13 \pm 1\%$ ($P < 0.01$) of the receptor capacity and testosterone a $29 \pm 6\%$ ($P < 0.02$) increase of the receptor affinity. A combination of estradiol-17β and testosterone did not modify the 1,25$(OH)_2D_3$ receptor's binding performances. In conclusion, the effects of corticoids, estradiol-17β and testosterone under *in vitro* conditions on the 1,25$(OH)_2D_3$ receptor's binding performances were found to be marginal in our system. Other studies under *in vivo* conditions, possibly at the pre-transcriptional level, of these steroids effects on the 1,25$(OH)_2D_3$ receptor gene regulation expression would be of great interest.

INTRODUCTION

1,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], the most active metabolite of vitamin D_3 interacts with a specific intestinal receptor [1, 2, 3] to mediate intestinal calcium absorption and *de novo* induction of an intestinal calcium binding protein [4] through a mechanism similar to that proposed for other steroid hormones [5]. It is generally assumed that the 1,25$(OH)_2D_3$ intestinal receptor exhibits only one ligand binding site with a dissociation constant calculated by the Scatchard method [6] to be in the range of $1–5 \times 10^{-10}$ M [7–13]. We have recently reported [14] the existence of positive cooperativity in the binding of 1,25$(OH)_2D_3$ to its chick intestinal receptor, and a biochemical characterization of this phenomenon in the 1,25$(OH)_2D_3$ intestinal chromatin receptor system [31]. The Hill plot [15] which takes account of this possibility of cooperativity, resulted in a much better fitting of the experimental data than the Scatchard model. Such a finding suggests that the Scatchard model is inappropriate [16].

Additionally, positive cooperativity has recently been characterized in two other steroid hormone receptor systems, namely estradiol [17] and progesterone [18].

The influence of steroid hormones, as glucocorticoids, estradiol-17β and testosterone on the vitamin D metabolism have recently become an important research area.

It is generally admitted [19] that glucocorticoid excess does not clearly affect plasma levels of 1,25$(OH)_2D_3$, but interacts with the terminal metabolism of 1,25$(OH)_2D_3$ at the intestinal level [20, 21] by a still unknown mechanism which results in a decrease of calcium absorption. Conflicting results have been reported on the effect of glucocorticoids on 1,25$(OH)_2D_3$ receptors in rat osteogenic cells and mouse bone cells in culture [22–29]; this receptor concentration was found to be increased in rat species [22, 23], while it was reduced in the mouse [24].

A regulation of the 1,25-dihydroxyvitamin D receptor levels in the chick oviduct shell gland following *in vivo* injections of diethylstilbestrol have already been reported [15] and a synergistic effect of estradiol-17β and testosterone on the stimulation by 1α-(OH)D_3 of calcium absorption and of duodenal calcium binding protein has recently been observed [26]. Also the presence of a receptor-like protein in rat yolk sac for 1,25$(OH)_2D_3$ has recently been reported [27] and another receptor-like protein for 1,25$(OH)_2D_3$ has been characterized in the rat testis [28].

All these observations strongly suggest the existence of interactions of these steroid hormones in the vitamin D endocrine system. However, all these interaction studies are the results of experiments conducted *in vivo* or in cell culture systems and make difficult to situate the interaction level of these steroid hormones at the pre or posttranscriptional level of the 1,25$(OH)_2D_3$ receptor synthesis. Here we would like to report the *in vitro* effects of a large excess (20,000 molar excess) of triamcinolone, estradiol-17β and testosterone on the 1,25$(OH)_2D_3$ binding performances to its chick duodenal chromatin receptor.

EXPERIMENTAL

Chemicals

[^{3}H]-1,25(OH)$_2$D$_3$ (sp. act. 85 Ci/mmol) was obtained from Amersham/Searle. 1,25(OH)$_2$D$_3$ was a kind gift from Dr Milan Uskokovic (Hoffmann-La Roche, Nutley, NJ). Triamcinolone acetonide, estradiol-17β-3-benzoate and testosterone–proprionate were obtained in crystallized form from Sigma Laboratories.

Animals

White Leghorn cockerels were obtained on the day of hatching (Pace-Setter, Anaheim, Calif.) and raised on a standard rachitogenic diet [29] (0.6% calcium; 0.4% phosphorus) for 2 weeks, *ad libitum*; then they were raised on a normal diet containing normal levels of calcium and phosphorus (1.2% Ca, 0.7% phosphorus) and 2000 IU of vitamin D$_3$ per kg of diet for two additional weeks.

Chromatin preparation

The chicks were killed by decapitation; the duodenal loop was excised, and its contents flushed out with saline solution. It was then slit longitudinally and washed with a saline solution (0.9% NaCl). All the subsequent steps were performed at 0–4°C. The mucosa was scraped from the serosa with the aid of two chilled glass slides on an inverted Petri dish over ice. Then the mucosa was homogenized in TED buffer (Tris 10 mM; EDTA 1.5 mM; dithiothreitol 1 mM; pH 7.4) [40% weight/volume] containing 300 μM phenylmethylsulfonyl fluoride (PMSF) and centrifuged at 5000 *g* for 10 min. The pellet containing the cell nuclei was resuspended in the same volume of TED-Triton (0.5%) buffer, vortexed and centrifuged at 5000 *g* for 10 min. Two additional washes with TED-Triton were followed by two other centrifugations. The final pellet was resuspended in the same initial volume of TED-PMSF.

Binding assays

Aliquots (100 μl) of the crude chromatin receptor preparations were incubated for 18–20 h at 0.4°C with increasing concentrations of [^{3}H]1,25(OH)$_2$D$_3$ ranging from 0.4 to 9.5 nM, in the presence or absence of a 200-fold excess of 1,25(OH)$_2$D$_3$ to determine both the specific and non-specific ligand binding. Other aliquots of the same chromatin preparation were incubated in the same condition with the same range of [^{3}H]-1,25(OH)$_2$D$_3$ concentrations, in the presence of a 20,000-fold excess of various steroid hormones (Triamcinolone, estradiol-17β, testosterone) for each 1,25(OH)$_2$D$_3$ concentration. The non-specific binding of 1,25(OH)$_2$D$_3$ in those conditions was determined by a parallel incubation in the presence of a 200-fold excess of both 1,25(OH)$_2$D$_3$ and the steroid hormone studied. The hormone bound to the receptor was then separated from the free ligand by the hydroxylapatite batch assay, as described previously [30].

Data treatment

The Hill coefficient (n_H) was calculated from the slope of the Hill plot. The Hill equation [15] describing a cooperativity between two binding sites is: $[B] = [B_{max}][F]^{n_H}/K_d + [F]^{n_H}$ where B is the ligand specifically bound to the receptor, F is the free ligand, n_H is the Hill coefficient and K_d is a composite average of the dissociation constants of the two binding sites of the receptor. Values of n_H greater than one indicate a positive cooperativity, and lower than one a negative cooperativity. In n_H equals one, the Hill equation simplifies to become the same formulation as the Scatchard equation.

Others

Protein concentrations were determined by the Biuret method.

RESULTS

Influence of a 20,000 *excess of triamcinolone on* 1,25(*OH*)$_2$*D*$_3$ *binding to its chick intestinal receptor*

The saturation analysis (see Fig. 1A) of the intestinal receptor by its ligand [^{3}H]1,25(OH)$_2$D$_3$ was not modified by the presence of triamcinolone. The Scatchard plot was concave downwards in the absence or in the presence of triamcinolone (Fig. 1B). The Hill plot (Fig. 1C) gave a much better fitting of the data. The Hill coefficient was unchanged ($n_H = 1.83$) and indicated a very high positive cooperativity in this receptor preparation. The presence of triamcinolone did not change the affinity and capacity of the receptor, as well as the cooperativity level (see Table 1) in four different batches of chromatin preparations. So, the presence of triamcinolone did not modify the 1,25(OH)$_2$D$_3$ receptor performances in this system.

Influence of a 20,000 *excess of estradiol-*17β *on* 1,25(*OH*)$_2$*D*$_3$ *binding to its chick intestinal receptor*

The presence of estradiol-17β shifted to the left the whole saturation curve of 1,25(OH)$_2$D$_3$ receptor (see Fig. 2A). However, this increase in the binding capacity was not significant at low concentrations of ligand. The cooperativity level was slightly, but not significantly reduced from 1.50 ± 0.12 to 1.33 ± 0.09 (see Table) and the affinity decreased from 1.03 ± 0.05 to 1.36 ± 0.25, which are the values of the receptor dissociation constants. However, this decrease was not significant. A $13 \pm 1\%$ ($P < 0.01$) increase of the maximum binding capacity was induced by the presence of estradiol-17β.

Influence of a 20,000 *excess of testosterone on the* 1,25(*OH*)$_2$*D*$_3$ *binding to its chick intestinal receptor*

The presence of testosterone shifted to the left the saturation curve of 1,25(OH)$_2$D$_3$ receptor (Fig. 3) at low concentrations only. However, this increase of the binding capacity at 1,25(OH)$_2$D$_3$ low concentrations was not significant. The cooperativity level

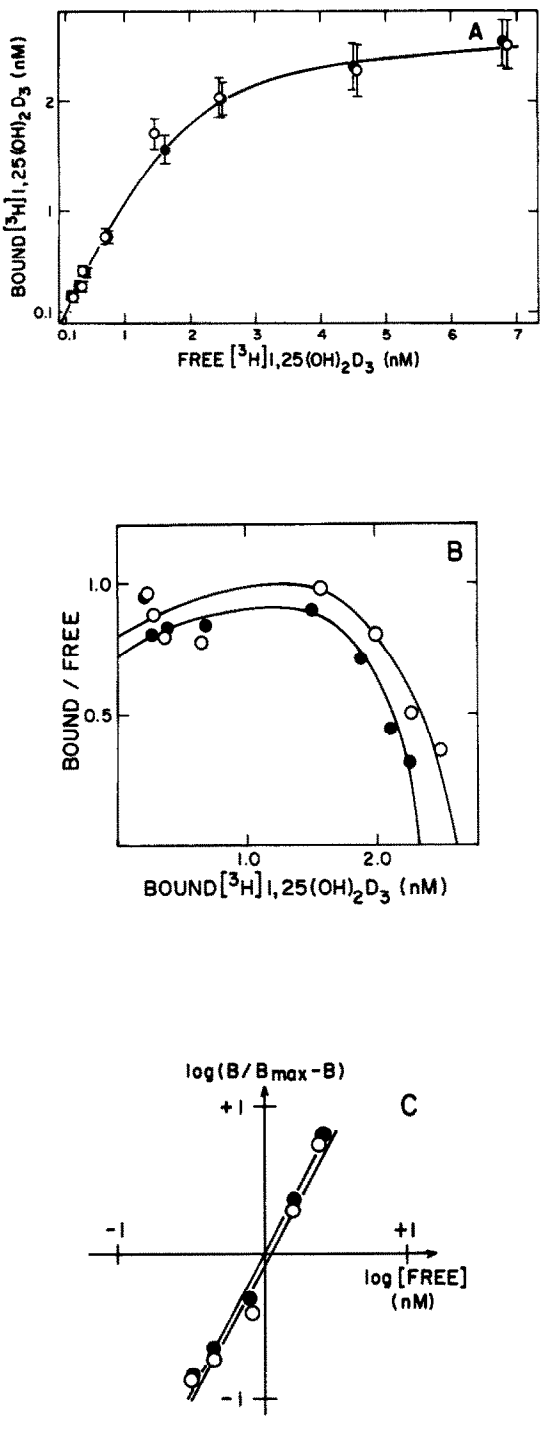


Fig. 1. Saturation analysis (A) of 4 different concentrated (40%) chromatin chick duodenal receptor preparations, each resulting of a 3-chick group raised on a normal diet supplemented with vitamin D_3, by increasing concentrations of $[^3H]1,25(OH)_2D_3$ in the presence or absence of a 200-fold excess of $1,25(OH)_2D_3$. Each point represents the mean ± SEM of the specific binding. Scatchard analysis (B) and Hill plot (C) of the saturation of the same chromatin preparation issued from one batch of 3 chicks. These analyses were performed in the absence (●——●) or in the presence (○——○) of a 20,000 molar excess of triamcinolone acetonide for each concentration of $[^3H]1,25(OH)_2D_3$.

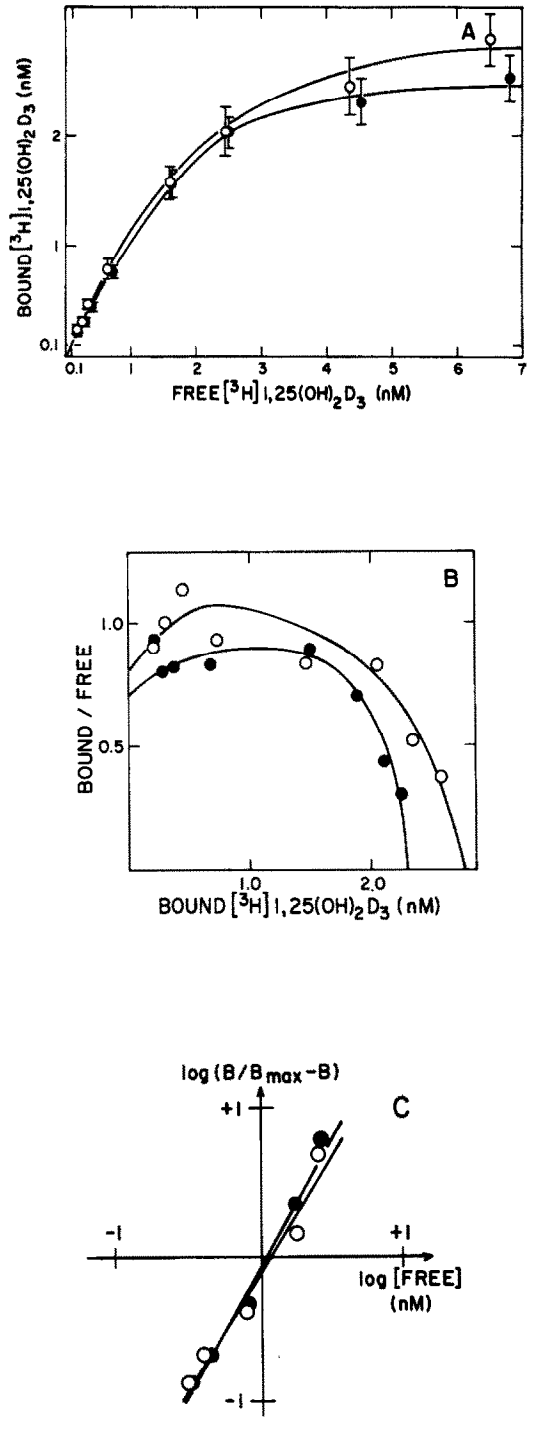


Fig. 2. Saturation analysis (A) of 4 different concentrated (40%) chromatin chick duodenal receptor preparations, each resulting of a 3-chick group raised on a normal diet supplemented with vitamin D_3, by increasing concentrations of $[^3H]1,25(OH)_2D_3$ in the presence or absence of a 200-fold excess of $1,25(OH)_2D_3$. Each point represents the mean ± SEM of the specific binding. Scatchard analysis (B) and Hill plot (C) of the saturation of the same chromatin preparation issued from one batch of 3 chicks. These analyses were performed in the absence (●——●) or in the presence (○——○) of a 20,000 molar excess of estradiol-17β-3-benzoate, for each concentration of $[^3H]1,25(OH)_2D_3$.

Table 1. Intestinal chromatin $1,25(OH)_2D_3$ receptor's binding performances in the absence or presence of a 20,000 molar excess of triamcinolone acetonide, estradiol-17β-3 benzoate, testosterone–propionate and both compounds for each concentration of [^{3}H]$1,25(OH)_2D_3$ studied

	K_d (nM)	n_H	B_{max}
Control	1.03 ± 0.05	1.50 ± 0.12	186 ± 15
Triamcinolone	0.97 ± 0.14	1.59 ± 0.10	183 ± 16
Estradiol-17β	1.36 ± 0.25	1.33 ± 0.09	212 ± 19
Testosterone	0.82 ± 0.05	1.53 ± 0.05	175 ± 22
Testosterone + Estradiol	1.00 ± 0.12	1.37 ± 0.07	172 ± 19

The indicated values are the mean $\pm$ SEM of 4 different batches of crude chromatin, each of them constituted of 3 chicks. K_d is the average dissociation constant of the receptor's two binding sites extrapolated from the Hill plot (in nM), n_H is the Hill coefficient and B_{max} is the maximum binding capacity in fmol/mg protein.

and the receptor capacity were unchanged (see Table 1). A $29 \pm 6\%$ significant ($P < 0.02$) increase of the receptor affinity was induced by the presence of testosterone; the receptor dissociation constants varied from 1.03 ± 0.05 nM to 0.82 ± 0.05 nM.

Influence of a 20,000 *excess of testosterone combined to a* 20,000 *excess of estradiol-17β*

The saturation curve of the $1,25(OH)_2D_3$ receptor was slightly, but not significantly shifted to the right (Fig. 4) by the presence of testosterone and estradiol-17β, especially at high concentrations of [^{3}H]$1,25(OH)_2D_3$. However, no significant change of the affinity, capacity and cooperativity level of the $1,25(OH)_2D_3$ receptor were induced by the simultaneous presence of these two steroid hormones (see Table 1).

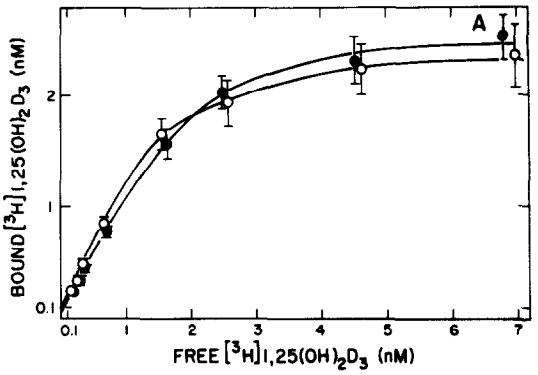
DISCUSSION

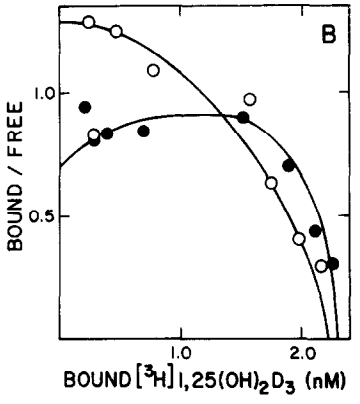
In our experiment, the effects of triamcinolone, estradiol-17β and testosterone, even in large molar excesses (20,000) on the $1,25(OH)_2D_3$ binding performances to the vitamin D repleted chick intestinal receptor, were found to be marginal, at best.

Triamcinolone acetonide did not have any effect on the affinity, cooperativity level and concentration (i.e. maximum binding capacity as expressed in fmol/mg protein) of the $1,25(OH)_2D_3$ receptor. So, a post-transcriptional action of glucocorticoids directly on the $1,25(OH)_2D_3$ receptor seems improbable. Other interactions at the pre-transcriptional level on the $1,25(OH)_2D_3$ receptor gene regulation's expression might be involved. This clearly needs further investigation.

Estradiol-17β increased slightly the receptor capacity without changing its affinity. However, this effect was certainly less dramatic than the observed regulations of $1,25(OH)_2D_3$ receptor levels in the chick oviduct shell gland, after injection *in vivo* of diethylstilbestrol [25]. Another effect of estradiol-17β at the $1,25(OH)_2D_3$ receptor gene level cannot also been excluded in this situation.

Testosterone induced a significant increase of $1,25(OH)_2D_3$ receptor affinity. This is, to our knowl-





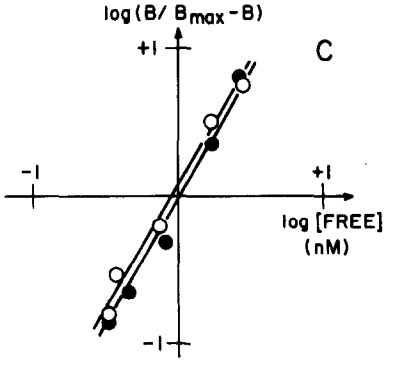
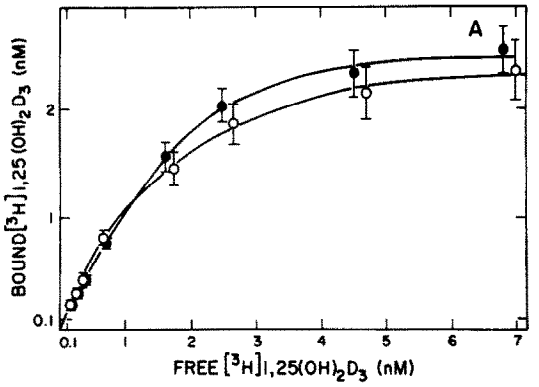
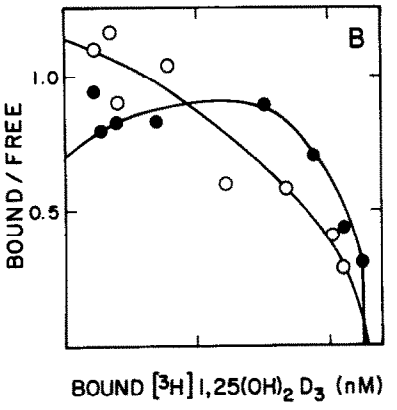


Fig. 3. Saturation analysis (A) of 4 different concentrated (40%) chromatin chick duodenal receptor preparations, each resulting of a 3-chick group raised on a normal diet supplemented with vitamin D_3, by increasing concentrations of [^{3}H]$1,25(OH)_2D_3$ in the presence or absence of a 200-fold excess of $1,25(OH)_2D_3$. Each point represents the mean $\pm$ SEM of the specific binding. Scatchard analysis (B) and Hill plot (C) of the saturation of the same chromatin preparation issued from one batch of 3 chicks. These analyses were performed in the absence (●——●) or in the presence (○——○) of a 20,000 molar excess of testosterone–proprionate for each concentration of [^{3}H]$1,25(OH)_2D_3$.





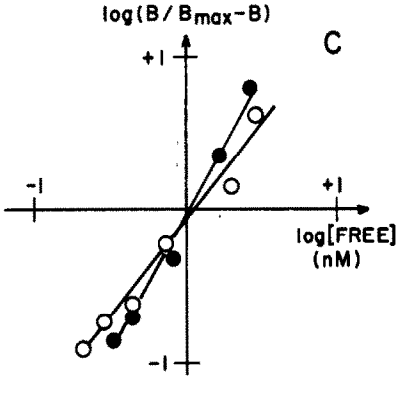


Fig. 4. Saturation analysis (A) of 4 different concentrated (40%) chromatin chick duodenal receptor preparations, each resulting of a 3-chick group raised on a normal diet supplemented with vitamin D_3, by increasing concentrations of [^{3}H]1,25(OH)$_2$D$_3$ in the presence or absence of a 200-fold excess of 1,25(OH)$_2$D$_3$. Each point represents the mean ± SEM of the specific binding. Scatchard analysis (B) and Hill plot (C) of the saturation of the same chromatin preparation issued from one batch of 3 chicks. These analyses were performed in the absence (●——●) or in the presence (○——○) of a 20,000 molar excess of estradiol-17β and testosterone, for each concentration of [^{3}H]1,25(OH)$_2$D$_3$.

edge, the first report of a direct effect of testosterone in the 1,25(OH)$_2$D$_3$ receptor's binding performances. Finally, the simultaneous presence of estradiol-17β and testosterone did not have any significant effect on the 1,25(OH)$_2$D$_3$ receptor binding performances.

In conclusion, all these observations do not support the possibility of a direct post-transcriptional action of glucocorticoids, estradiol-17β and testosterone on the binding performances of 1,25(OH)$_2$D$_3$ to its chick intestinal receptor. Additional studies at the pre-transcriptional level are needed in an attempt to elucidate the interaction mechanism(s) of steroid hormones on the vitamin D endocrine system.

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