CYCLIC UPTAKE OF STEROIDS IN CELLS AND CELL NUCLEI FROM PROSTATE, LIVER AND PITUITARY

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1. Introduction

Although an impressive knowledge has now accumulated concerning the binding of steroid hormones to cytosolic receptors, considerably less is known about the uptake and action of steroids in cell nuclei.

During the course of our studies on specific binding of the androgenic steroid [^{3}H]methyltrienolone in human benign hyperplastic prostate [1], we have investigated the uptake of the steroid by purified nuclei. Since we were unable to obtain steady-state levels of steroid–receptor complexes in the nuclei with time, we decided to study specific uptake of steroids into cells and cell nuclei from various steroid-dependent tissues. These studies which are reported below have indicated a cyclic occurrence of steroid–receptor complexes in prostate, liver and pituitary.

2. Materials and methods

2.1. *Preparation and incubation of nuclei*

Cell nuclei were prepared from frozen (−70°C) human benign hyperplastic prostate tissue essentially by the technique in [2]. Human prostate tissue was minced with a pair of scissors and homogenized with a teflon–glass homogenizer in 5 vol. Tris–HCl buffer (10 mM Tris, 1.5 mM $CaCl_2$, 1 mM $MgSO_4$, 0.25 M sucrose, pH 7.4). The homogenate was filtered through 2 layers of gauze and centrifuged at 800 × *g* for 10 min. The supernatant was discarded and the pellet was washed once with the same buffer. Following centrifugation at 800 × *g* for 10 min, the pellet was resuspended in Tris–HCl buffer (1 ml/0.5 g original tissue) (10 mM Tris, 1.5 mM $CaCl_2$, 1 mM $MgSO_4$, 0.88 M sucrose, pH 7.4). This suspension was layered on top of a discontinuous gradient (from the bottom of the tube: 1.7 ml 2.2 M sucrose, 1.7 ml 1.8 M sucrose and 1.8 ml 0.88 M sucrose solution containing the nuclear suspension). Following centrifugation at 105 000 × *g* for 45 min, the pellet was resuspended in the incubation medium (10 mM Tris, 50 mM NaCl, 5 mM $MgSO_4$, 0.05 mM EDTA, pH 7.4) and washed once in this buffer prior to incubation. The cell nuclei appeared pure under the light microscope.

For incubation, 0.2 ml of the nuclear suspension, containing 4.2 × 10^6 cell nuclei were added per tube. Varying concentrations of labelled hormones were added in 0.1 ml incubation buffer per tube. Parallel incubations were carried out with labelled hormone plus a 100-fold excess of unlabelled hormone for estimation of non-specific binding, allowing calculation of specific binding (total binding minus non-specific binding). In all cases triple assays were performed. After incubation for various times and temperatures, nuclei were sedimented at 6700 × *g* for 5 min and the supernatant (incubation medium) was analyzed with respect to protein-bound hormone following treatment with dextran-coated charcoal. 0.5 ml dextran-coated charcoal suspension (0.5%

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charcoal, 0.05% dextran T70, 0.1% gelatin) was added per tube and the mixture was shaken well with a Vortex and kept for 20 min at 0°C; after centrifugation at 6700 × *g* for 5 min, 0.6 ml was taken from the supernatant and measured for radioactivity. Nuclei were washed 5 times with 0.5 ml Tris–HCl buffer (10 mM Tris, 50 mM NaCl, 5 mM $MgSO_4$, 0.05 mM EDTA, pH 7.4) (each wash was checked for presence of bound [^{3}H]methyltrienolone) and were finally resuspended in 0.5 ml 0.8 M NaCl and kept for 1 h at 0°C. Following centrifugation at 6700 × *g* for 5 min, the whole supernatant was measured for radioactivity; the nuclear pellet was extracted with 0.5 ml acetone for 1 h at 0°C. After centrifugation, the supernatant was taken for measurement of radioactivity.

Non-specific binding was found to be ~70% for the incubation medium, 25–30% for the NaCl fraction and 35–40% for the acetone fraction.

Apparent K_a and B_{max} values were calculated with the help of a Wang programmable electronic calculator (Model 720).

2.2. *Preparation and incubation of liver and pituitary cells*

Liver cells were prepared essentially as in [3,4]. The cells were suspended in Leibowitz L-15 medium supplemented with 0.78 mM sucrose, 10 mM glucose, benzylpenicillin (60 mg/ml), 1% male rat serum and insulin (1.2 × 10^4 mU/l) and were added to petri dishes (8 × 10^5 cells/dish). The cells were incubated at 37°C with 5 × 10^{-8} M [^{3}H]dexamethasone or with 5 × 10^{-8} M [^{3}H]dexamethasone plus a 100-fold excess of unlabelled dexamethasone. At the end of the incubation, the petri dishes were cooled to 0°C and the cells washed 3 times with ice-cold Leibowitz L-15 medium. The cells were then scraped off in 1.5 mM $MgCl_2$–phosphate buffer (pH 7.4) solution (0.6 ml/petri dish) with a rubber tube. The cell suspension was homogenized with a Teflon–glass homogenizer and the homogenate centrifuged at 6700 × *g* for 5 min. The supernatant was analyzed for total and bound radioactivity using the dextran-coated charcoal technique. The pellet was washed twice with 10 mM Tris–HCl (pH 7.4) then extracted with 1 ml ethanol. The supernatant fractions and pellet extract were measured for radioactivity. Bound hormone was calculated as the sum of specifically bound steroid in the supernatant and in the pellet.

Pituitary cells were prepared as in [5]. The cells were suspended in Dulbecco's modified Eagle's medium (DMEM) and were added to petri dishes (8 × 10^5 cells/dish). Incubations were performed with 10^{-8} M [^{3}H]methyltrienolone or with 10^{-8} M [^{3}H]testosterone. Incubations with radiolabelled steroid in the presence of a 100-fold excess of unlabelled steroid were carried out in parallel. At the end of the incubation, the petri dish was cooled to 0°C, the medium was removed and the cells were washed 5 times with ice-cold DMEM. The cells were then scraped off the dish in ice-cold DMEM and aliquots of the cell suspension were counted for radioactivity. Each point represents the mean of 3 determinations.

3. Results and discussion

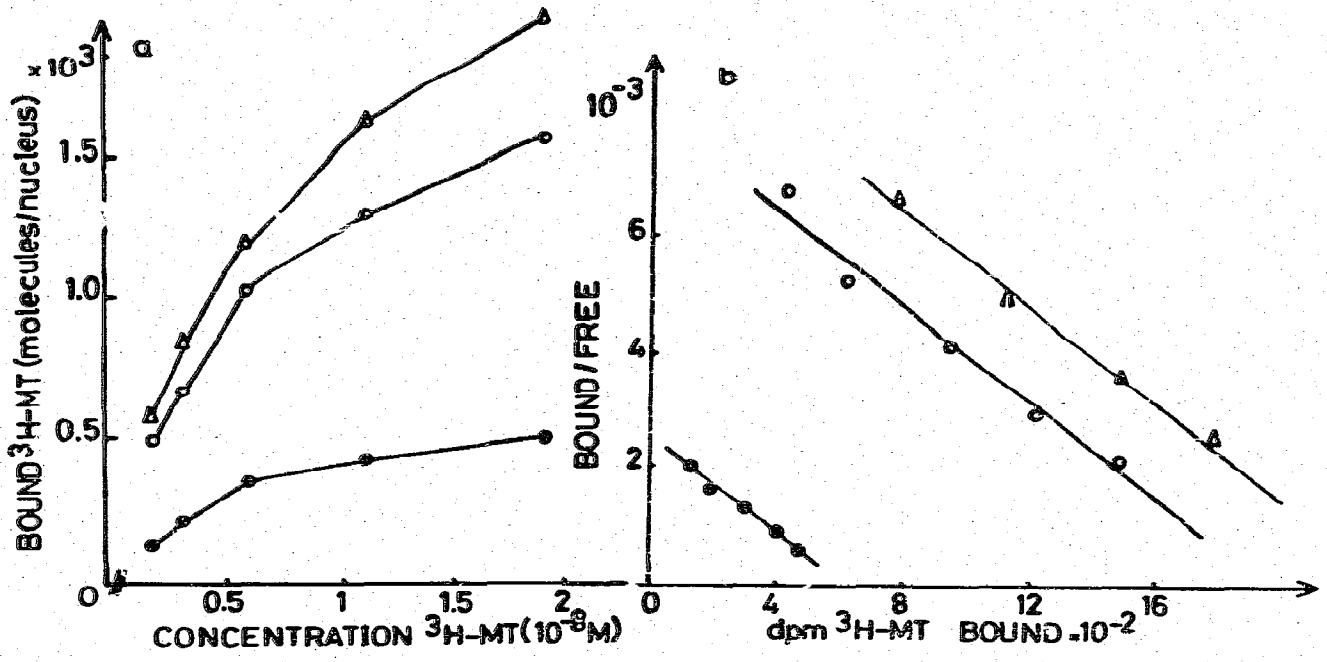
The uptake of [^{3}H]methyltrienolone by cell nuclei was found to be a saturable phenomenon (fig.1). Three types of steroid–receptor complexes were extractable from the nuclei:

1. Buffer-soluble complexes;
2. NaCl-soluble complexes;
3. NaCl-resistant–acetone-extractable complexes.

The buffer-soluble complexes were found in the incubation medium surrounding the nuclei. The NaCl-soluble complexes are probably loosely bound to nuclear structures whereas the acetone-extractable complexes appear to be more tightly bound. As can be seen from fig.1, the saturation curves for the NaCl-extractable and NaCl-resistant complexes appear very similar.

Scatchard analysis of the specific binding of steroid in incubation medium and in nuclei revealed that all the steroid–receptor complexes had an association constant of the same order of magnitude (fig.1). The maximal amount of binding sites was different in the 3 fractions as will be discussed below.

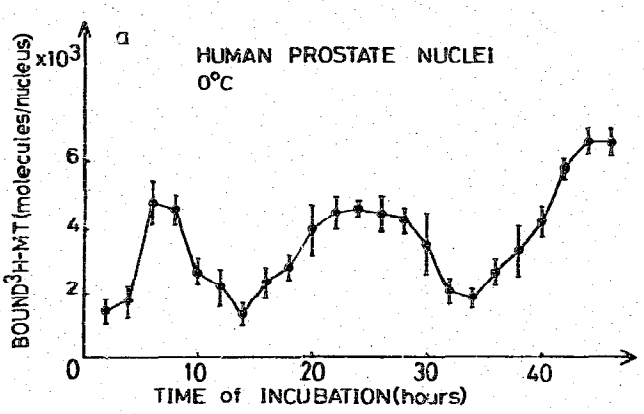
Using the same assay procedure, the time dependency of nuclear uptake of [^{3}H]methyltrienolone was studied at 0°C. The steroid concentration used was 7 × 10^{-9} M. As can be seen from fig.2a, specific binding of [^{3}H]methyltrienolone in prostate nuclei showed a peak following 6–8 h incubation. This was followed by a decreased concentration of bound

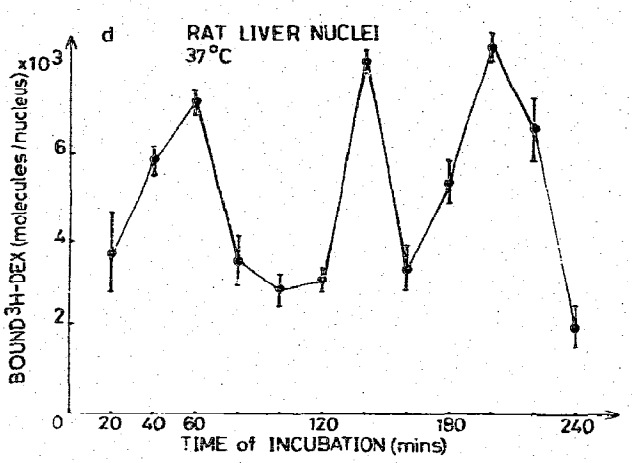


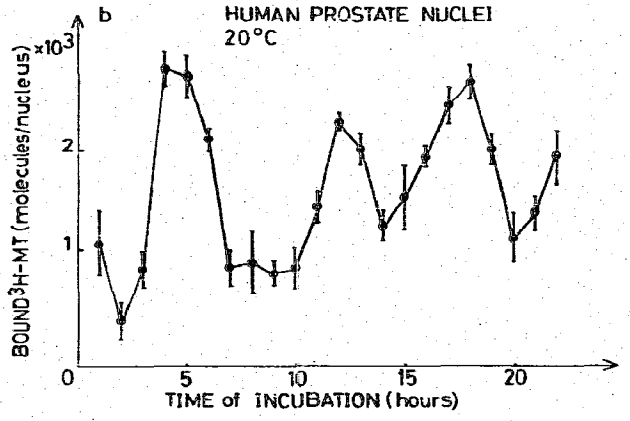
	buffer soluble complex	NaCl soluble complex	acetone extractable complex
association constant ± S.E.	3.2 ± 0.31 .10^8	3.5 ± 0.17 .10^8	3.8 ± 0.36 .10^8
Bmax (n/nucleus) 95% of confidence limit	1045 842 - 1404	3510 3245 - 3994	2870 2496 - 3526
correlation coefficient	-0.986	-0.998	-0.987

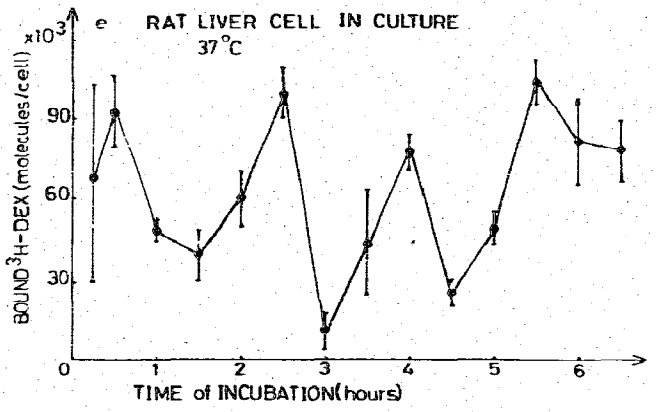
Fig.1. Uptake of [^{3}H]methyltrienolone in cell nuclei from human benign hyperplastic prostate tissue. Nuclei were prepared as described in section 2.1. They were incubated for 20 h at 0°C with varying concentrations of [^{3}H]methyltrienolone (0.1–1.9 × 10^{-8} M). (△–△–△) specifically bound, NaCl-extractable radioactivity; (○–○–○) specifically bound, acetone-extractable radioactivity; (●–●–●) specifically bound radioactivity in the incubation medium. Maximum binding capacity (B_{max}) is expressed as no. molecules specifically bound/cell nucleus.

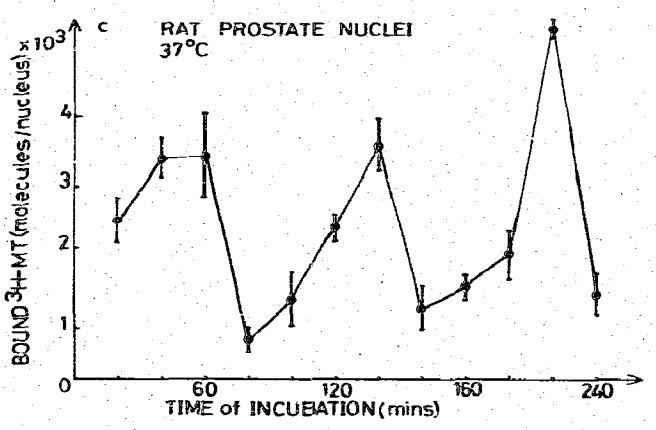
steroid in the nuclei with the lowest amount at 14 h incubation. A second peak of specific binding of radioactive ligand was observed at ~24 h incubation, followed by a second decline in concentration and a third peak at 44 h incubation. The same type of time-dependent cyclic specific uptake of ^{3}H-labelled steroid in human prostate nuclei was observed at 20°C (fig.2b) and in rat prostate at 37°C (fig.2c). Furthermore, cyclic uptake of [^{3}H]dexamethasone was observed in rat liver nuclei at 37°C during incubation of isolated nuclei with steroid (fig.2d) and in rat liver cells during incubation of steroid with isolated hepatocytes in tissue culture (fig.2e). Finally, cyclic uptake of steroid in tissue cultured cells was also

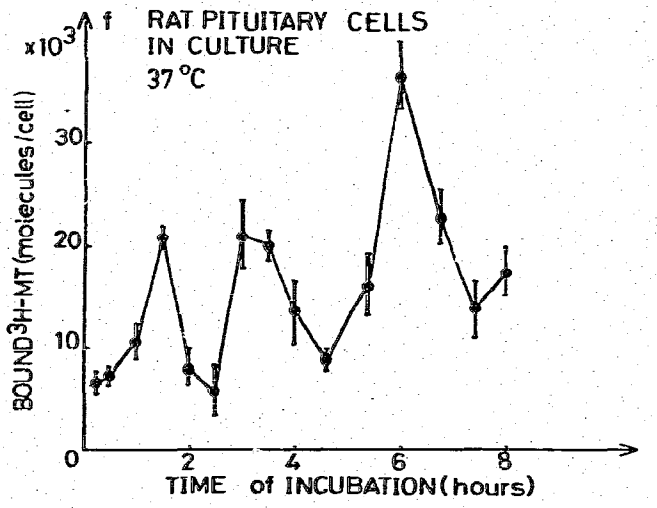

a
HUMAN PROSTATE NUCLEI
0°C
BOUND 3H-MT (molecules/nucleus) x10^3
TIME of INCUBATION (hours)


d
RAT LIVER NUCLEI
37°C
BOUND 3H-DEX (molecules/nucleus) x10^3
TIME of INCUBATION (mins)


b
HUMAN PROSTATE NUCLEI
20°C
BOUND 3H-MT (molecules/nucleus) x10^3
TIME of INCUBATION (hours)


e
RAT LIVER CELL IN CULTURE
37°C
BOUND 3H-DEX (molecules/cell) x10^3
TIME of INCUBATION (hours)


c
RAT PROSTATE NUCLEI
37°C
BOUND 3H-MT (molecules/nucleus) x10^3
TIME of INCUBATION (mins)


f
RAT PITUITARY CELLS
IN CULTURE
37°C
BOUND 3H-MT (molecules/cell) x10^3
TIME of INCUBATION (hours)

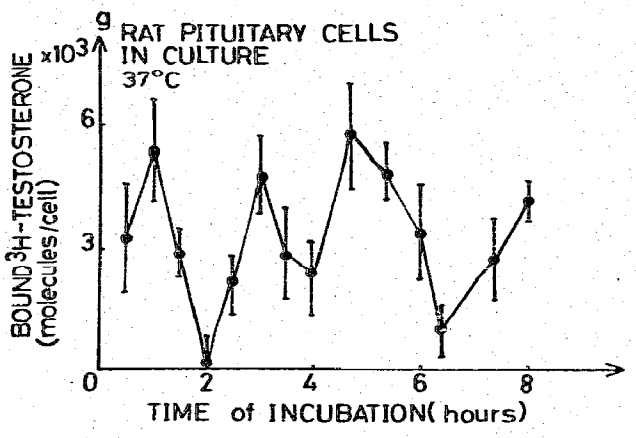


Fig.2. Time-dependent specific uptake of ^{3}H-labelled steroid in nuclei from prostate and liver and in liver and pituitary cells. The experiments illustrated in fig.2(a–d) were performed with nuclei purified as described in section 2.1. Incubations were carried out with 7×10^{-9} M [^{3}H]methyltrienolone ([^{3}H]MT) (prostate nuclei) or 3×10^{-8} M [^{3}H]dexamethasone (liver nuclei). Bound hormone is the sum of specifically bound steroid found in the incubation medium and extracted from nuclei with 0.1 M NaCl and acetone. Each point represents the mean of 3 determinations ± 1 SD. The experiments illustrated in fig.2(e–g) were performed with liver and pituitary cells as described in section 2.2.

obtained during incubation of [^{3}H]testosterone (fig.2f) and [^{3}H]methyltrienolone (fig.2g) with pituitary cells.

From the data presented in fig.2, it is evident that the length of the cycles of nuclear steroid–receptor complexes is temperature-dependent. At 0°C, a full cycle takes 18 h, at 20°C it decreases to 7 h and at 37°C it is about 1.5 h. Cyclic specific uptake of steroid hormones is observed in prostate, liver and pituitary tissue with both natural and synthetic steroid hormones under saturation conditions. Furthermore, cyclic uptake of steroids occurs both in nuclei in cell-free systems and in intact cells. The results from the experiments with hepatocytes and pituitary cells argue against the possibility that the cyclic uptake of steroid observed with isolated prostate cell nuclei at saturating concentration of steroid is an artifact associated with this cellfree system.

It was shown [1] that the [^{3}H]methyltrienolone–receptor complex in human prostate cytosol is rapidly degraded at 37°C. The relative temperature-resistance of the nuclear androgen–receptor complexes described in the present study contrasts markedly to this lability. A relatively high temperature-resistance for the estrogen–receptor complex in chick oviduct nuclei has been reported [6]. It should be kept in mind, however, that the cell-free system used in the present investigation is complex. The specific binding depends on the kinetics of association of the labelled ligand to the receptor, of the dissociation of the endogenous hormone from the receptor and of the inactivation of the receptor.

The steroid–receptor complex found in the medium during incubation with nuclei may be functionally related to the NaCl-extractable and NaCl-resistant forms of the steroid–receptor complex, since it always occurs in parallel with these forms. It is also possible that it represents a nuclear complex that has diffused out from the nuclei during the centrifugation procedure following incubation. The existence of a similar release of receptor–hormone complex from cell nuclei has been reported for the thyroid hormone receptor [7].

Our results on the NaCl-extractable and the acetone-extractable steroid–receptor complexes in nuclei are in agreement with reports on the existence of two different binding sites for the steroid–receptor complexes in cell nuclei [8,9]. It was suggested [9] that the steroid–receptor complex, after translocation to the nucleus, first appears as a unit loosely bound to chromatin and later becomes more firmly attached to this structure. These authors have also proposed that the NaCl-resistant binding sites in the nucleus may represent specific acceptor sites.

The molecular mechanisms underlying the cyclic occurrence of specific binding of [^{3}H]methyltrienolone in cell nuclei from prostate, liver and pituitary may only be speculated upon. It seems possible that the nuclear receptor passes through regular conformational cycles, possibly steroid-induced, and that only one type of conformation allows high-affinity, low-capacity binding of the steroid.

A more detailed experiment was also performed by measuring the distribution of [^{3}H]methyltrienolone binding to buffer-, NaCl- and acetone-extractable fractions after incubation of prostatic nuclei at 20°C for various times. The data presented in fig.3 show that steroid binding does not occur at the same time in the three fractions. Non-specific binding (fig.3b) was almost constant during the first 14 h incubation,

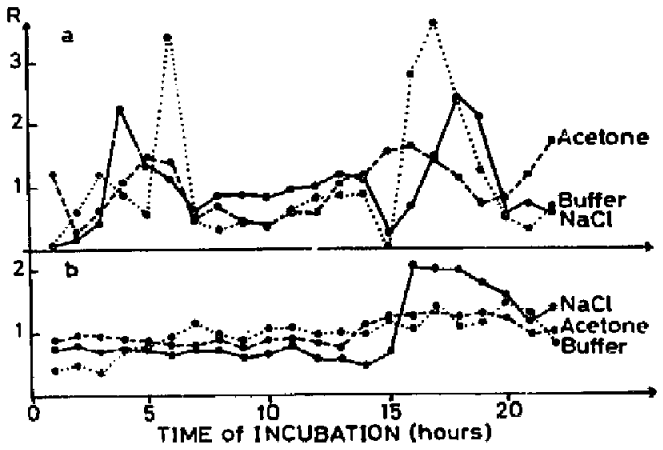


Fig.3. Relative changes of specific (a) and non-specific (b) binding found in buffer (●···●)-, NaCl (●——●)- and acetone (●---●)-extractable fractions of human prostatic nuclei. R = ratio between the radioactivity in a specific fraction and the mean radioactivity calculated for all points of one type of extract. The experiment was carried out as in fig.2b.

and was then increased in the NaCl-extractable fraction. Since the non-specific binding was ⩽ 30% of the total binding it did not influence the measurement of specific binding.

In order to obtain cyclic uptake of steroids, we have found it necessary to use:

(i) An optimal number of cell nuclei (to avoid aggregation);
(ii) A steroid concentration that saturates the binding sites of the receptor;
(iii) repeated washings (5 times) of the cell nuclei to remove all free steroids.

A possible model for receptor recycling in cell nuclei based on the results obtained here is given in table 1. The rates of the proposed reactions are probably different. It may be speculated that the most energy-consuming step, in the presence of an excess of steroid, in the suggested scheme is the translocation of the receptor. Our model for receptor recycling in cell nuclei would therefore seem to be in consonance with the rate-limiting translocation of steroid receptors in cell nuclei suggested [10].

The biological importance of the cyclic occurrence of steroid receptors in cell nuclei is at present obscure. It may, however, represent a phenomenon of great importance for the understanding of steroid–receptor interactions with chromatin and may cast light upon processes as receptor recycling and the so-called 'off reaction'. The cell-free experiments indicate that

Table 1
A model of receptor recycling

								Radioactivity detected		
								Incubation medium	NaCl extract	Acetone extract
I	[SR]	+	DNA	→	[SR]	~	DNA	–	+	–
II	[SR]	~	DNA	→	SR	+	DNA	–	–	+
III	[SR]	–	DNA	→	R	≃	DNA + S	–	–	–
				+S*						
IV	R	≃	DNA	→	[S*R]	≃	DNA	–	+	+
		↓				↓				
				+S*						
V	R	+	DNA	→	[S*R]	+	DNA	+	–	–

The steroid–receptor complex (SR) first binds to nuclear components (designated 'DNA') to form an NaCl-soluble extract (I) and subsequently binds stronger to form an acetone-extractable complex (II). In the next step (III), the receptor undergoes a conformational change, possibly in connection with a translocation, or as a result of a change in the structure of chromatin, and loses its ligand (S). The steroid-binding site on the receptor is then restored and the radioactive tracer (S*) may bind to receptor molecules attached to DNA (IV) and the receptor molecules (R) that have dissociated from DNA (V). More work is needed to establish the validity of this hypothetical scheme. Since radioactivity detected in the same type of extract may belong to different steps in the reaction scheme, consecutive changes in amount of buffer-, NaCl- and acetone-extractable radioactivity will not be observed

synthesis of new protein is not a requisite for the cyclic uptake of steroid into nuclei.

Our results may be taken as an indication that results from quantitation of nuclear receptors with the steroid exchange procedure [11] should be interpreted with due caution and with consideration of the cyclic character of nuclear steroid uptake typical of at least some steroid-responsive tissues.

Acknowledgements

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