EXTRACTION, PARTIAL PURIFICATION AND CHARACTERIZATION OF 'THE INSOLUBLE ESTROGEN RECEPTOR' FROM CHICK LIVER NUCLEI

M. C. LEBEAU, N. MASSOL and E. E. BAULIEU

*Unité de Recherches sur le Métabolisme Moléculaire et la Physio-Pathologie des Stéroides de l'Institut National de la Santé et de la Recherche Médicale, Université Paris-Sud, Département de Chimie Biologique, Hôpital de Bicêtre, 78 rue du Général Leclerc, 94270-Bicêtre, France**

Received 1 April 1974

1. Introduction

When purified nuclei from chick liver are suspended in a cytosol or buffer solution of ^{3}H-labelled oestradiol, or when liver tissue is incubated in buffer containing the radioactive hormone, 80% of the ^{3}H-labelled oestradiol bound to the nuclei is recovered in a protein fraction which cannot be extracted by the normal high salt or detergent solutions. These and other data reported previously [1] led to the definition of an 'insoluble' *receptor.*

This paper reports the solubilization of the *receptor* by treatment of purified nuclei with dilute trypsin at 10°C. In its soluble form, the *receptor* has binding parameters and specificity similar to those determined in intact nuclei, and its physico-chemical characteristics have been analyzed by gel filtration and sucrose gradient ultracentrifugation in order to compare it to the readily soluble *receptors* from chick liver [2] and other organs [3].

2. Materials and methods

2.1. *Materials*

Steroids, enzymes, counting and DNA and protein measurements were all as described in ref. [1]. Slight modifications are the following: both laying hens and 3–4 week-old immature chicks were White Leghorns, and trypsin was Calbiochem (bovine pancreas) grade A.

* Postal address: Lab Hormones, F 94270 – Bicêtre.

2.2. *Preparation and extraction of nuclei*

'Chauveau' type nuclei were prepared in 2.4 M sucrose, 5 mM Tris, 3 mM $MgCl_2$, pH 7.4, as described previously [1] method 1). They were then washed in 0.25 M sucrose–Tris–Mg^{2+}, centrifuged at 700 *g* and the pellet extracted successively in:

(a) 0.005 M Tris–3 mM $MgCl_2$, pH 7.4: four homogenizations followed by 700 *g* centrifugations, in order to remove soluble nuclear proteins.

(b) 0.01 M Tris–1.5 mM EDTA–0.5 M KCl, pH 7.4: homogenization, freezing 1–15 hr at –20°C, thawing followed by a 105 000 *g* centrifugation, to extract the soluble nuclear receptor [2].

(c) Treatment by trypsin 0.15 mg/ml [4] in 0.25 M sucrose–Tris– Mg^{2+}, 15–30 min at 10°C followed by 105 000 *g* centrifugation, to extract the 'residual' proteins [1].

(d) four extractions with methanol.

In a typical experiment, nuclei from 5 g of liver tissue and corresponding to 6 mg of DNA (2.7×10^9 nuclei based on the value of 2.3 pg DNA/nucleus) are extracted four times with 3 ml Tris–Mg^{2+} (a), once with 2 ml Tris–KCl (b), then with 3 ml trypsin solution (c) and four times with 1 ml methanol (d).

2.3. *Incubation with the hormone*

In a series of preliminary experiments, in order to follow the extraction process, purified whole nuclei were incubated for 30 min at 37°C with ^{3}H-labelled oestradiol (1 nM in 0.25 M sucrose–Tris–Mg^{2+}, pH 7.4), washed and extracted according to procedures (a) through (d), and aliquots of the super-

natants were counted. To quantify the extraction of the nuclear binding proteins, the solubilized fractions (a) to (c) were incubated overnight at 2°C with 4 nM ^{3}H-labelled oestradiol then treated with a charcoal–dextran suspension [1] for 1 hr to remove the free hormone, and centrifuged at 2000 *g* for 5 min. To test the specificity of the various fractions, identical aliquots were simutaneously incubated with ^{3}H-labelled oestradiol in the presence of non-radioactive oestradiol 1 μM. Aliquots of the supernatant containing the bound hormone were counted and specific activities (bound dpm/mg DNA or protein) were determined.

When binding parameters of the soluble proteins were to be measured, the extracts (a) to (c) were incubated for 20 hr at 2°C with concentrations of radioactive hormone varying from 0.1 nM to 1 nM, and for specificity studies with 0.2 nM oestradiol in the presence of 50-fold concentrations of non-radioactive potential competitors. Free steroid was then removed by the charcoal–dextran technique and binding was plotted according to Scatchard.

3. Results

3.1. *Extraction of nuclear binding proteins*

When nuclei are incubated with 1 nM ^{3}H-labelled oestradiol and then washed and extracted as described under Materials and methods (a) to (d), only 13–15% of the total nuclear radioactivity can be extracted by Tris–0.5 M KCl (b), whereas trypsin action liberates 50–60% of these counts (c) (table 1). An extra 50% of the remaining radioactivity can be extracted by repeating the enzyme treatment, and it is probable that progressively nearly a 100% extraction could be reached in this manner. Charcoal treatment shows that most of the extracted radioactivity in solutions (b) and (c) is in the unbound form. When the soluble extracts are secondarily incubated overnight at 0–2°C in the presence of 4 nM ^{3}H-labelled oestradiol (i.e. 'reloaded' with hormone), then many binding sites are detected by the charcoal technique and approximately ten times more bound hormone is found in the trypsin – (c) than in the KCl – (b) extract (table 2). The protein ratio in the extracts is 3:1 respectively, which indicates that the enzyme treatment extracts more binding protein than the high-salt solution.

Table 1
Extraction of ^{3}H-labelled oestradiol from chick liver nuclei

No. of experiments	0.5 M KCl	Trypsin	Methanol
10	14.4		85.3
9		55.4	43.9
3	13.0	34.6	52.3
	Average amount of protein in each fraction (mg protein/g fresh tissue).		
	0.5	1.3	0.9

Nuclei from 5 g liver tissue were incubated with 1 nM ^{3}H-labelled oestradiol in 2.5 ml 0.25 M sucrose, 5 mM Tris, 3 mM $MgCl_2$, pH 7.4, 30 min at 37°C, washed 4 times with 3 ml incubation buffer or Tris–Mg^{2+}, and extracted by the indicated solutions ((b) to (d) in the text). Results are expressed as percentages of extracted radioactivity, which represents 30–45% of the incubated radioactivity. Successive treatments are written from left to right.

Table 2
Extraction of oestradiol-binding proteins from chick liver nuclei

Tris (a)	0.5 M KCl - extract (b)	Trypsin extract (c)
–	8000	73 350
+	5578	66 073

The sequential extractions described in the text (a) to (c) were studied. Binding was not quantified in the hypotonic Tris extracts (a) and therefore this step is described only as having (+) or not having (–) taken place. KCl- and trypsin-extracts (b) and (c) were incubated with ^{3}H-labelled oestradiol 4 nM for 20 hr at 2°C, treated 1 hr with a charcoal–dextran suspension, and the bound cpm in the supernatant were counted. Results from a typical experiment are expressed in dpm bound per mg DNA (corresponding to 4×10^8 nuclei). Other experiments confirm the fact that 20–40% of the KCl extractable binding sites could be extracted by hypotonic Tris.

One can see in table 2, that a preliminary hypotonic Tris extraction (a) of the nuclei, reduces the quantity of binding protein recovered in the KCl-extract (b). For this reason, and because the soluble binding protein is in smaller quantity than the trypsin-extractable protein, the double extraction (a) + (b) did not seem necessary, and in experiments designed to study the properties of the trypsin extract

step (b) was left out. Another reason for skipping (b) is that high salt concentrations seem to inhibit oestradiol binding to the trypsin-extractable receptor (unpublished results). This is not yet clearly understood and will be commented upon in another paper.

3.2. *Trypsin action*

The effect of temperature on the extraction of nuclear binding proteins by a trypsin solution was tested. The temperature 10°C was then chosen because more binding sites were extracted than at 4°C, and no loss of protein binding capacity was observed during a 30 min incubation at this temperature.

A time course of trypsin action showed that a plateau was reached after incubating the nuclei 10 min in the presence of the enzyme, and that the binding was unaltered for 30 min (fig. 1) A control of trypsin activity in the extract showed that the enzyme activity was decreased in the presence of nuclei (fig. 2); this might explain the limited proteolysis observed, and the resulting preservation of intact oestradiol binding.

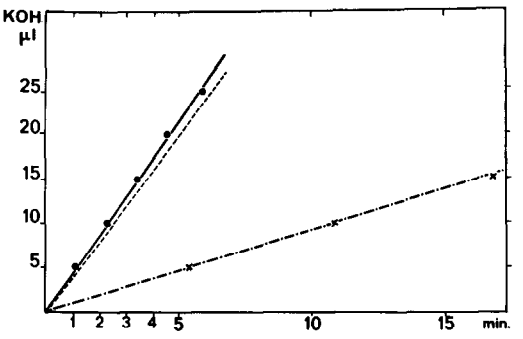

Fig. 2. Trypsin activity in the presence of chick liver nuclei: 0.2 M KOH is added to maintain pH = 8.0 in the presence of 50 µg enzyme and 25 ml of 10^{-3} M α-*N*-benzoyl-l-arginine ethylester (BAEE) in 0.2 M KCl; (●——●) in 10^{-3} N HCL; (– – – –) in 0.25 M sucrose, Tris, Mg^{2+}; (×—·—×) in the presence of trypsin extract from liver nuclei (in 0.25 M sucrose, Tris, Mg^{2+}).

3.3. *Characteristics of the solubilized nuclear receptor*

The binding protein is sensitive to pronase, and not to DNAase, RNAase or phospholipase A. It has a dissociation constant $K_D = 1.89 \pm 0.28$ (SD) x 10^{-10} M (8 det.) as calculated according to Scatchard (fig. 3), and one finds approximately 100 sites per cell* in a

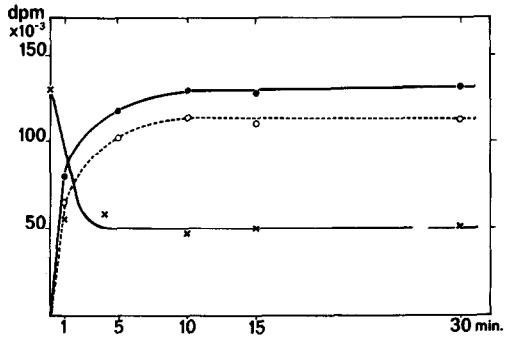

Fig. 1. Time course of solubilization of the trypsin-extractable nuclear receptor. Nuclei are incubated with trypsin (0.15 mg/ml) at 10°C: (●——●) radioactivity bound in the trypsin extract (step c, see text) after reloading with 1 nM ^{3}H-labelled oestradiol for 20 hr at 2°C and 1 hr charcoal treatment; (o– – –o) radioactivity specifically bound in the trypsin extract: same as above, after substraction of the radioactivity bound in equivalent extracts reloaded with 1 nM ^{3}H-labelled oestradiol in the presence of 1 µM oestradiol and treated with charcoal; (×——×) radioactivity recovered after trypsin extraction in the pellets (step d) of nuclei previously incubated with 1 nM ^{3}H-labelled oestradiol.

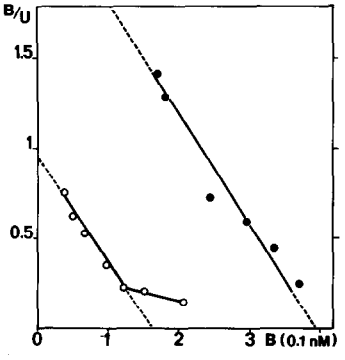

Fig. 3. Binding of ^{3}H-labelled oestradiol to the trypsin extractable nuclear receptor. Trypsin extracts from immature chick (o——o) and laying hen (●——●) nuclei were incubated with various concentrations of ^{3}H-labelled oestradiol for 20 hr at 2°C. Immature chick : protein concentration = 1.55 mg/ml, 84 binding sites/cell, $K_D = 1.62 \times 10^{-10}$. Laying hen : protein concentration = 1.64 mg/ml, 346 binding sites/cell, $K_D = 1.63 \times 10^{-10}$ M.

* The number of sites measured on the Scatchard plot are calculated per corresponding DNA and divided by 2.3 pg DNA/cell [5].

15-day old chick (male or female) and 3–5 times that quantity in laying hens or immature chicks having received a subcutaneous injection of oestradiol (20 mg/kg) 24 hr beforehand. Hormone specificity is roughly the same as that observed in whole nuclei (table 3).

Table 3

Competitor	Decrease of binding in the presence of the competitor
Oestradiol	83
Diethylstilboestrol	81
Oestrone	53
17 α-Oestradiol	28
Oestriol	11
Testosterone	0
Progesterone	0

Results are decrease of binding of 0.2 nM ^{3}H-labelled oestradiol in the presence of 10 nM competitor, expressed as a percentage of the control.

Ultracentrifugation analysis on a 5–20% sucrose gradient in 0.01 M Tris 1.5 mM EDTA showed the protein to have a sedimentation coefficient of approximately 4 S. The presence of 0.5 M KCl in the gradient buffer did not change significantly this migration. The high salt extractable nuclear binding protein (KCl extract) had the same sedimentation coefficient (fig. 4a). When filtrated on Sephadex G-200 in 0.01 M Tris–1.5 mM EDTA pH 7.4, a 5-fold purification was observed when measured as specific activity (number of binding sites/mg protein). 85% of the bound radioactivity was eluted in the '4 S region' (i.e. the trypsin - or calcium-treated 4 S oestrogen receptor from calf uterus cytosol was eluted in the same volume on calibrated Sephadex G-200 columns [6,7] and fig. 4b), the remaining 15% appearing in the void volume and may be corresponding to break-through elution of the *receptor*. Stokes radius measured on a calibrated Sephadex G-200 column is 35.5 Å, very close to that of bovine serum albumin.

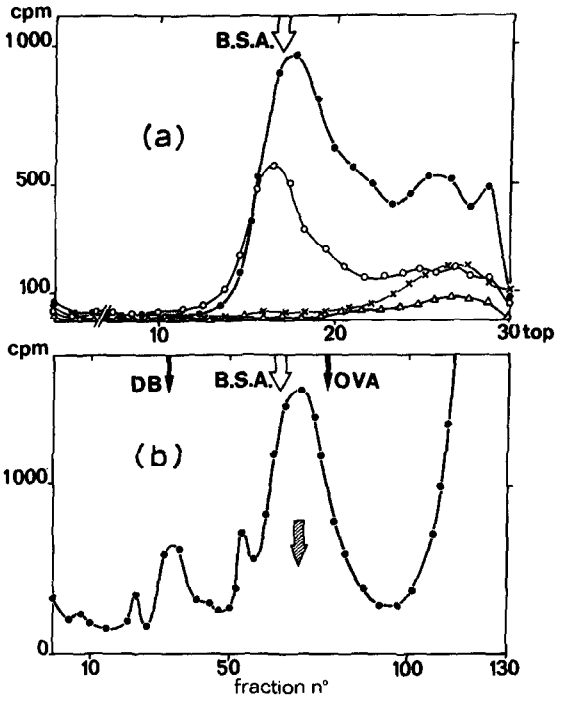

Fig. 4. Ultracentrifugation and gel filtration of the trypsin-extractable nuclear *receptor*. (a): ultracentrifugation in a 5–20% sucrose gradient in Tris–EDTA–0.5 M KCl, Beckman rotor SW 56, 16 hr at 45 000 rpm. All extracts were pre-incubated 20 hr with radioactive hormone with or without non-radioactive competitor, and treated 1 hr with charcoal–dextran before ultracentrifugation: (•——•) trypsin extract, 0.5 nM ^{3}H-labelled oestradiol; (o——o) KCl extract, 0.5 nM ^{3}H-labelled oestradiol; (×——×) trypsin extract, 0.5 nM ^{3}H-labelled oestradiol + 1 μM oestradiol; (△——△) KCl extract, 0.5 nM ^{3}H-labelled oestradiol + 1 μM oestradiol. (b): Elution of trypsin extract from a Sephadex G-200 column (2.5 × 36 cm) in 0.01 M Tris–1.5 mM EDTA. The shaded arrow indicates the elution volume of 4 S cytosol *receptor* from calf uterus (see text), and radioactivity after fraction 100 is free ^{3}H-labelled oestradiol. BSA: bovine serum albumin, Ova: ovalbumin, D.B.: Dextran Blue.

4. Discussion

This communication reports the solubilization of the heretofore 'insoluble' nuclear oestrogen *receptor* from chick liver nuclei. Extraction by high salt or detergent solutions had been tried unsuccessfully; recent experiments show that dilute trypsin solubilizes at least 50% of the strongly attached protein, which once extracted in this manner, retains the oestrogen binding properties observed in intact nuclei. Hormone specificity and sensitivity to hydrolytic enzymes are similar, the *apparent* affinity of the solubilized receptor for oestradiol is 5-10 times higher than that measured in

the residual pellets after incubation at the nuclear level. This difference can be explained if one considers that changes in environment may modify the *receptor's* physico-chemical properties; kinetics applied to soluble binding systems are different from those described for heterogenous systems (where diffusion–reaction kinetics introducing diffusion coefficients and substrate fluxes, must be used instead of Michaelis–Menten kinetics [8]. Or more simply, methods used to measure the binding parameters in the soluble or the insoluble systems are different and can bring about these variations.

Relationships that may exist between KCl-extractable and trypsin extractable binding proteins are difficult to establish. Are these two *receptors* the same protein, its two main 'states' being due to physiological conditions (e.g., the presence or the absence of oestradiol; experiments devised to demonstrate this have been negative) or to the localisation of the receptor (the soluble form loosely bound to chromatin or in a hydrophilic environment, the insoluble form much more strongly attached or in a hydrophobic environment).

The trypsin-extractable *receptor* behaves much like the trypsinated 4 S form of calf uterus cytosol oestrogen *receptor* [7] on gradient or on Sephadex G-200 columns and when analyzed by ultracentrifugation on sucrose gradients. Although in this laboratory no well-defined cytosol oestrogen *receptor* was found in chick liver [2], these similarities suggest that all these proteins might belong to a same class, and that their localisation could be species- or cell-cycle dependent.

No clear explanation can be given for the manner in which trypsin solubilizes the 'insoluble' nuclear *receptor*. It is tempting to compare this action with the results obtained in this and other laboratories showing that whereas crude or partly purified calf uterus cytosol *receptor* binds to homologous chromatin or DNA in vitro, trypsin-treated cytosol does not ([9], T. Erdos and A. Alberga, personnal communications). This suggests that the *receptor* protein has an oestradiol binding site, unaltered by trypsin, and a sequence enabling it to bind to DNA (or another nuclear component), which is cut off by enzyme action. The chick liver nuclear *receptor* could possess such a structure, and trypsin could detach it from its strong association with chromatin by nicking off a polypeptide section bound to DNA. However, the enzyme solubilizes many other proteins along with the *receptor* (compare in table 1, the protein concentrations of the various extracts), and this effect is obviously not selective.

References

[1] Lebeau, M.C., Massol, N. and Baulieu, E.E. (1973), Eur. J. Biochem. 36, 294.
[2] Mester, J. and Baulieu, E.E. (1972) Biochim. Biophys. Acta 261, 236.
[3] Advances in the Biosciences, (1971) 7, (G. Raspé, ed.) Pergamon Press.
[4] Pommier, J., de Prailauné, S. and Nunez, J. (1972) Biochimie, 54, 483.
[5] Common, R.H., Chapman, D.G. and Maw, W.A., (1951) Can. J. Zool., 29, 265.
[6] Erdos, T., Benada, R., Best-Belpomme, M., Fries, J., Gospodarovicz, D., Menahem, M., Reti, E. and Veron, A. (1971) in: Advances in the Biosciences (G. Raspé, ed.) 7, 119, Pergamon Press.
[7] Olga Soulignac, (1973) Thèse de Médecine, Paris,
[8] Selegny, E., Brown, G., Geffroy, J., and Thomas, D. (1969) J. Chim. Phys. et Physico-chimie Biol. 66, 391.
[9] André, J. and Rochefort, H. (1973) FEBS Letters, 32, 330.