PURIFICATION OF ESTRADIOL *RECEPTOR* BY AFFINITY CHROMATOGRAPHY. REPRESENTATIVE EXPERIMENTS

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Received 30 June 1973

1. Introduction

Progress in the understanding of steroid hormone actions awaits the purification of specific high affinity binding proteins (*receptors*: 'R') present in the soluble (high speed supernatant) fraction of the target tissue homogenates [1].

The purification of the uterine estradiol *receptor* [2] involves two major difficulties. The concentration, estimated on the basis of one binding site per molecule, indicates that a purification of at least 5×10^4 from the cytosol is necessary. Furthermore, the *receptor* has, under various conditions, different molecular forms, particularly almost irreversible aggregates. On the other hand, the high affinity of the *receptor* for estradiol (E_{II}) (K approx. 10^{10} M^{-1}) and the very slow dissociation rate of the complex at 0°C (k_{-1} approx. 10^{-5} sec^{-1}) [3] suggest that it might be easily separated from the bulk of the proteins by affinity chromatography [4]. Preliminary studies and considerations of the binding parameters of the *receptor* and other (non specific) proteins, indicated that a purification of the order of 10^3 was possible in one such step.

The use of affinity chromatography columns (estradiol-2 or 4-diazo-*p*-amino-benzylcellulose) [5] and (estradiol-17α-n-propyl thiol-*N*-phenylene-maleimide-polyvinyl) [6] have led, according to the published reports, to the removal of 70–90% of the *receptor* from uterine soluble preparations. However no *receptor* was recovered from these columns. This result has been generally attributed to a difficulty in removing tightly bound protein or to some molecular alteration of the *receptor*. In fact, *receptor* is not fixed by the previously mentioned columns, but is occupied and therefore masked by either on covalently bound ligand, 'adsorbed' on the polymeric support at the time of the chemical linkage (G. Puca, personal communication and [7]) or alternatively by ligand released from a covalent link during chromatography [8]. Occupied *receptor* is therefore eluted subsequently from the column, and is not detected. It follows that methods for measuring binding sites in the presence of non radioactive ligand had to be developed [7] and used to study the binding capacity of chromatographed extracts. New affinity chromatography adsorbents have been synthesized [9] and the necessity of extensive washing treatment by organic solvents is now recognized.

Here are reported a series of representative results of purification of calf uterus estradiol *receptor* obtained with two satisfactory adsorbents.

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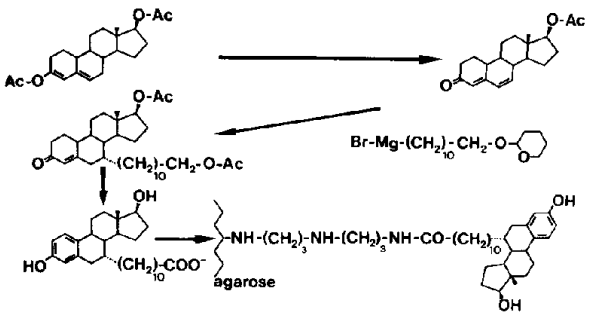

Fig. 1. Synthesis of agarose-7 α adsorbent.

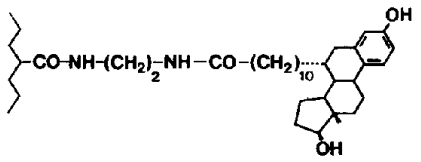

Fig. 2. Acrylamide-7 α adsorbent.

2. Materials and methods

2.1. *Biological material*

Calf uteri were obtained immediately after slaughter and transported on ice. They are trimmed, cut into small pieces and homogenized in a Braun mixer (5 × 10 S) at maximum speed in Tris 10 mM, EDTA 1.5 mM, pH 7.4 buffer ('Tris'), 2 ml/g of tissue. The homogenate was centrifuged 700 × 20 *g*/min. The supernatant was centrifuged 45 000 × 150 *g*/min. To this high speed supernatant were added KCl and $CaCl_2$ to final concentrations of 0.4 M and 4 mM respectively and then left for 60 min at 0°C [10]. This crude extract is called 'cytosol'. Although the biological significance of the form of the *receptor* obtained under these conditions is still obscure, this '4 S' form has been chosen because of its relative homogeneity and its pH_i approx. 7.

2.2. *Binding measurements*

'Direct' determinations of available binding sites were performed after incubation at 0–4°C until equilibrium (approx. 12 hr) with estradiol 30 nM. The high affinity bound radioactive estradiol was measured by the 3 following techniques.

2.2.1. 'Differential dissociation'

Free and rapidly dissociating radioactive estradiol was removed by incubation for 4 hr with a charcoal 0.5%, dextran T 80 0.50% suspension in 0.01 M Tris, 0.015 M EDTA, and 0.4 M KCl, pH 7.4 ('Tris KCl') (vol/vol). Such a technique is reliable for measuring specific binding provided there is sufficient protein concentration (> 1 mg protein/ml) [11].

2.2.2. Hydroxyapatite adsorption of the *receptor*

The *receptor* was adsorbed on hydroxyapatite microcolumns which were then extensively washed at 0°C to remove free and easily freed ligand [12].

2.2.3. Gel filtration

A 0.5 ml sample was filtered on a coarse Sephadex G-25 10 ml column at 0°C (flow rate 40 ml/hr). The latter two techniques can be used with diluted extract without the loss of activity which occurs with charcoal when the concentration of proteins is less than 1 mg/ml.

2.3. *'Exchange' determinations*

When a non radioactive ligand occupies a part of the binding sites, in order to measure the total binding sites, under standard conditions, it is necessary first to saturate all of the sites, then, after removing the unbound ligand to exchange the bound fraction for radioactive hormone.

Thus extracts were first incubated until equilibrium with 100 nM non radioactive estradiol, excess ligand removed at 0°C using hydroxyapatite columns or charcoal adsorption and the exchange performed at 25°C for 2.5–4 hr in presence of a large excess of radioactive hormone.

[^{3}H]estradiol was obtained from CEA, Saclay, France (S.A. of 50 Ci/mole). Radioactivity was measured by counting a 0.5 ml sample in the Bray's solution at an efficiency of 16%.

Proteins were measured by the Folin–Lowry technique after dialysis against large volumes of water at neutral pH.

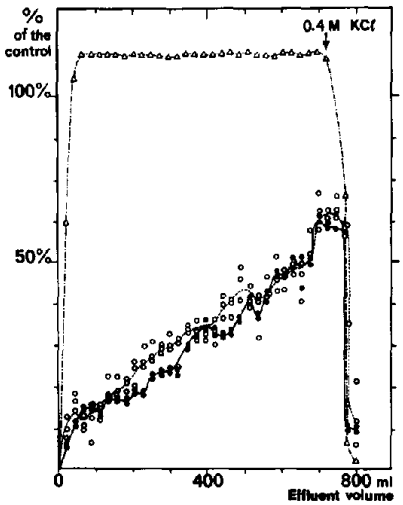

Fig. 3. Estradiol binding activity and proteins in the effluent of a 2 ml column during the application of the sample (720 ml of cytosol) and the initial washing (see table 2). 23 ml Fractions were collected. Free binding sites (●—●—●) and total binding sites (○---○---○) were measured by charcoal technique. Proteins were estimated at A_{280nm} (△---△---△). The arrow indicates the beginning of the washing.

3. Results

3.1. *Synthesis and preparation of 2 suitable adsorbents*

3.1.1. Agarose 7α-estradiol derivative ('agarose-7α')

An undecyclenic arm was fixed at the level of carbon 7 of the steroid nucleus by conjugate addition of the magnesium derivative to the dienic ketone form of the steroid [13]. Treatment of the crude condensation product with HCl, acetylation and purification by chromatography on silica gel gave the pure 7α-isomer (fig. 1). Oxidation with lead tetraacetate provoked aromatisation of ring A. The subsequent alkaline cleavage of the acetate groups and the selective benzoylation of the phenolic hydroxy group allowed the use of chromic acid for oxidizing the primary alcohol of the chain to a carboxyl group. The ketone, concurrently formed at the C 17 level, was reduced and the ring A benzoate was saponified to give the 3 17 β-dihydroxyestra-1,3,5 (10)-trien-7α-undecanoic acid. The affinity of this derivative for the cytosol *receptor* is of the order of 10^7 M^{-1}. Finally the estradiol derivative was coupled to dipropylamino agarose [4] obtained from Sepharose 4B, by the action of i-butylchloroformate in aqueous dioxane in the presence of tri-n-butylamine. Extensive washing followed with ethyl acetate–water (1:1) and dioxane–water (1:1) mixtures.

Table 1
Washing and elution of a 7α-agarose column.

	Total activity (cpm)
Wash 1 0°C	260 000
Wash 2 0°C	50 000
Wash 3 30°C	67 000
Wash 4 30°C	200 000
Wash 5 30°C	80 000
Eluate 30°C + E_{II} 20 μM*	1 320 000

* This value is corrected to the initial specific activity of 50 C/mM E_{II}.

Volume of the column: 2 ml washes and elution were done with 10 ml Tris–KCl. For experimental details see 2.2 and 2.3.

3.1.2. Polyacrylamide 7α-estradiol derivative ('acrylamide-7α')

The same estradiol derivative was coupled to aminoethyl polyacrylamide (from Biogel P 300) by the same procedure (fig. 2). Titration of free amino groups indicates less than 50 mEq/g adsorbent. Washes were performed with water, ethyl ether and dioxane.

3.2. *Attachment of the receptor to the column by affinity mechanism*

A 2 ml agarose-7α column was equilibrated with Tris–KCl, charged with 720 ml of cytosol and subsequently washed by 100 ml of the same buffer at 0°C. The *receptor* content of effluent fractions was identical whether it was measured directly or by an exchange technique (fig. 3), indicating that agarose-7α was not contaminated by adsorbed ligand and did not release it during this initial step of chromatography. Moreover, the comparison of protein and *receptor* profiles showed a rapid saturation of the protein adsorption capacity whereas the *receptor* attachment capacity was not yet saturated, suggesting a specific mechanism for *receptor* uptake. However part of the *receptor* is bound to such a column non specifically. The estradiol binding activity of successive washes and the eluate of an agarose-7α column (see details in 3.3.) are presented in table 1. These results suggest

Table 2
Purification of cytosol *receptor* by agarose 7 α-column.

	Crude cytosol	7 α-agarose eluate
Volume (ml)	720	25
Protein (mg/ml)	12	0.06
RECEPTOR ACTIVITY (bound E_{II})		
dpm/ml	315 500	637 500
pmoles/ml	2.84	5.74
Total pmoles	2 025	143
SPECIFIC ACTIVITY		
dpm/mg Protein	26 000	10 600 000
pmoles/mg Protein	0.23	94.40
PURIFICATION		408
RECOVERY		
From cytosol*		7%
From column*		12%
From column + rinsing buffer**		15%

* After elution exchange at 30°C.
** After elution exchange at 30°C and additional rinsing at 30°C. In this experiment 60% of the *receptor* was bound to the column (1 215 pmoles)

that a part of the *receptor* may be bound to the column by ionic exchange interactions.

3.3. *Partial purification of the receptor*

After charge and Tris–KCl washing (3.3 and fig. 3), the agarose-7 α column was treated batchwise with 20 ml of the Tris–KCl buffer. After centrifugation, the pellet was incubated at 30°C in 10 ml of Tris–KCl containing 1 mC of 5 Ci/mmole [^{3}H]estradiol.

After 60 min, the adsorbent was separated from the eluate by centrifugation and rinsed by 5 ml Tris–KCl buffer at 30°C. Both the eluate and the rinsing buffer were cooled at 0°C and filtered through a 100 ml Sephadex G-25 column (flow rate 100 ml/hr) to eliminate part of the large excess of estradiol and salt. Binding activity was measured in the void volume fractions by the hydroxyapatite adsorption technique. The overall results are summarized in table 2.

Purification has also be obtained using the 7 α-acrylamide column (fig. 2). Attachment of the *receptor* to this adsorbent is generally lower (50%) than to the agarose under standard conditions, but the purification is greater.

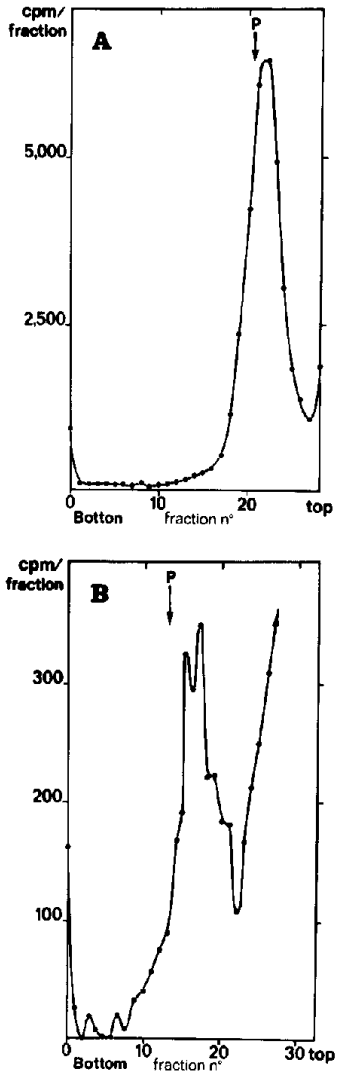

Fig. 4. Ultracentrifugation at 2°C of *receptor* purified on acrylamide-7 α column (linear sucrose gradient, 5–20% in Tris buffer). 0.2 ml Samples were applied; Two drop fractions were collected: A) cytosol (47 000 rpm × 12 hr); B) eluate (initial activity 7 500 cpm/ml – 61 000 rpm × 16 hr 30 min). Arrow represents the sedimentation of peroxydase (internal marker).

3.4. *Characterisation of the eluted binding protein as receptor*

Estradiol binding protein purified from either agarose or acrylamide 7 α-derivative columns has been analysed by sucrose gradient ultracentrifugation, Sephadex G-200 filtration and isoelectric focusing.

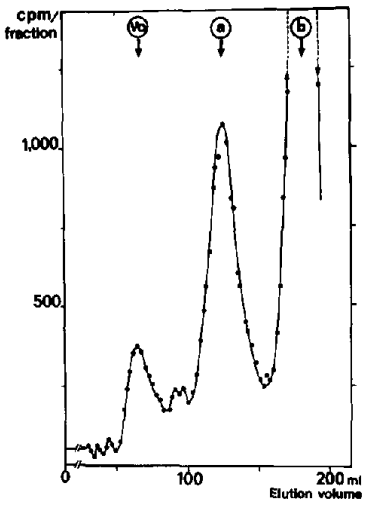

Fig. 5. Sephadex G-200 filtration. A 3 ml sample of the eluate from a acrylamide-7 α column (7500 cpm/ml) is applied on a 170 ml column equilibrated with Tris buffer. Elution was performed at 4°C with Tris buffer at a 10 ml/hr flow rate. 1.75 ml Fractions were collected: V_0 = void volume; a = Ca^{2+} treated *receptor;* b=free estradiol.

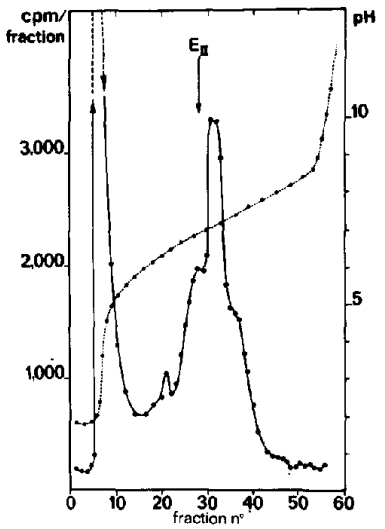

Fig. 6. Isoelectric focusing. A 0–50% sucrose gradient containing 2% ampholines pH 5–8 was prepared in a LKB-110 ml apparatus. A 6 ml sample (eluate from agarose-7 α column – activity 10 000 cpm/ml) was applied. The gradient was allowed to focus until the amperage fell to a constant 1 mA at 700 V. The column was refrigerated at 0°C. 2 ml Fractions were collected and pH measured at 0°C. Under our conditions E_{II} standards focused in a very sharp peak at the position indicated by the arrow (whether focused, alone or mixed ([^{14}C]estradiol with the eluate).

Sucrose gradient analyses (fig. 4) indicate that the binding entity of the acrylamide 7 α-column purified extract labelled by radioactive estradiol, sediments identically to that in the initial crude cytosol. A similar result was obtained with the agarose-7 α purified preparation, although the *receptor* was less homogeneous, some additional 4–6 S and heavier binding components being found. The G-200 filtration results (fig. 5) were almost identical to those obtained by ultracentrifugation for the two adsorbents. On the contrary electro focusing profiles show no significant difference between eluates from the two columns (fig. 6). The peak of bound estradiol is eluted at pH 7.2 as in crude cytosol. In addition a large peak of radioactivity is obtained in early fractions (pH < 6).

4. Discussion

Evidence has been presented for a significant purification by affinity chromatography of the cytosol estradiol *receptor.* The major problem of contamination by polymer-adsorbed ligand has been minimized. However problems still remain, particularly the low recovery from the column (10–40%). This cannot be attributed to an insufficient amount of exchangeable estradiol during the elution. The apparent loss of *receptor* may be due to two factors, either to some unknown 'interaction' with the adsorbent, or the decreased stability of the protein when it is highly purified. Indeed, recent modifications of the adsorbent (e.g. acetylation of residual free amino groups, in order to minimize the ionic exchange properties of the column) have decreased the non specific interactions, and therefore significantly increased (more than 2-fold) the purification. The evaluation of various materials and the basic techniques for exchanging ligands [7], a number of new adsorbents and the ligand affinities for the *receptor* [8], and a series of purification experiments [14] will be soon reported in detail.

The present results represent major advances. The highest specific activity obtained in one step chromatography with these adsorbents (acrylamide-7 α) is 167 pmoles/mg protein, whereas the best published results, by classical techniques [10], have been 55 pmoles/mg protein. It appears that an even better purification can be obtained with our adsorbents, particularly by the modifications of the matrix mentioned

above. Other selective adsorbents to purify the same *receptor* have been recently proposed [15, 16].

Experiments under way, using in addition the classical methods for protein fractionation are also promising in this respect. Moreover, whereas in the present work, the purified 4 S form seems identical to the starting component found in the crude cytosol, the purification of other molecular forms has still to be performed.

Acknowledgements

This work has been partially supported by the Ford Foundation and the Délégation Générale à la Recherche Scientifique et Technique.

The authors thank Doctor Walter Desmond for help in writing the manuscript and Anne Atger and Danielle Prod'homme for secretarial assistance.

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