PARTIAL PURIFICATION AND CHARACTERIZATION OF PROGESTERONE-BINDING PROTEIN FROM PREGNANT GUINEA PIG UTERUS

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Summary: After in vitro labeling with radioactive progesterone, a steroid macromolecule complex was isolated from the cytosol fraction of pregnant guinea pig uterus. The binding protein was purified about 200-fold by chromatographies on hydroxylapatite, DEAE-cellulose and Sephadex G-200. The following molecular characteristics were assessed for the binding protein: Sedimentation coefficient 5 S, Stokes radius 5.5 nm, apparent molecular weight 106,000, pI 2.5, and association constant for progesterone 3.2×10^9 l/mole. Relative affinity for ligands was as follows:progesterone >5α-pregnane-3,20-dione>deoxycorticosterone>5β-pregnane-3,20-dione>20β--hydroxy-4-pregnen-3-one>5α-dihydrotestosterone>testosterone. No significant affinity for pregnenolone, 17α-hydroxyprogesterone,cortisol or estradiol was detected. Neither the crude cytosolic steroid-macromolecule complex nor the partially purified progesterone-binding protein formed aggregates in a low salt medium. No splitting of the macromolecule into subunits was observed in high ionic media, either.

Studies aimed to clarify the mechanism of action of certain steroid hormones have revealed that specific binding of steroids to "receptors" of target organs is one of the first cellular events taking place in this process. Specific binding proteins for estradiol-17β, 5α-dihydrotestosterone, cortisol and aldosterone have been found in their respective target tissues (1-8). Concerning progesterone, binding to macromolecules has been found in the uteri of some mammalian species (9,10),and a receptor protein has been partially purified and characterized from a non-mammalian source, oviduct cytoplasm of estrogen-treated chicks (11,12).

During pregnancy, the progesterone-binding capacity of guinea pig blood plasma is increased 100-fold over the level in the non-pregnant animal (13-15). This enhanced progesterone-binding ability seems to be due to a specific macromolecule different from plasma cortisol-binding protein (14,15). We here describe a progesterone-binding protein in uterine tissue of pregnant guinea pigs, apparently different from that found in blood plasma of pregnant animals and from that induced by estrogen-priming in uteri of non-pregnant guinea pigs.

MATERIAL AND METHODS

Steroids. 1,2-^{3}H-Progesterone (specific activity 50 Ci/mmole and 1,2,6,7-^{3}H-progesterone (107 Ci/mmole were purchased from the Radiochemical Centre, Amersham, United Kingdom. Unlabeled steroids were obtained from Steraloids (Pawling, N.Y., U.S.A.) or Ikapharm (Ramat-Gan, Israel). All the reference compounds were purified prior to use with chromatography on hydroxyalkoxypropyl Sephadex (16).

Preparation of uterine cytosol fraction. Uteri and blood were taken from guinea pigs on the 55th-65th day of pregnancy. Before decapitation, the animals were anesthetized with ether and bled by cardiac puncture. Uterine horns were carefully freed from endometrium and surrounding fat, and the myometrial samples were thoroughly washed several times with 3 volume samples of Tris-EDTA buffer (10 mM Tris, 1.5 mM EDTA, 3 mM NaN_3, pH 7.4). Tissue samples were minced with scissors and homogenized in the same buffer with a Potter-Elvehjem type homogenizer (30% homogenate). After centrifugation at 105,000 x g_{max} for 90 min, the resulting supernatant was collected (uterine cytosol fraction) and used as starting material for purification.

Purification procedure. After overnight incubation at +4^{o}C with labeled progesterone (ca. 50 ng), 100 ml of uterine cytosol was chromatographed in two separate portions on a hydroxylapatite column (Hypatite, Clarkson Chemical Co., PA., U.S.A., column dimensions 30 x 420 mm) previously equilibrated with 10 mM potassium phosphate buffer, pH 7.4. The column was eluted with potassium phosphate by increasing the molarity stepwise at a flow rate of about 100 ml per hour. The fractions containing the bulk of the radioactivity were pooled, concentrated by ultrafiltration and passed for the second time through a hydroxylapatite column with 10 mM potassium phosphate as the eluting buffer. The most active fractions were combined, concentrated by ultrafiltration and subjected to gel filtration on Sephadex G-200 (Pharmacia, Uppsala, Sweden, column dimensions 26 x 640 mm) equilibrated with the Tris-EDTA buffer. Elution was effected by constant hydrostatic pressure, using the upflow technique, at a flow rate of 8 to 10 ml per hour. In order to estimate molecular Stokes radii (17), the column was calibrated with blue dextran, potassium dichromate, bovine serum albumin and yeast alcohol dehydrogenase. The fractions of highest specific activity were combined and this pool was used for further characterization of the progesterone-binding macromolecule.

Analytical methods. Sucrose density gradient centrifugations were performed in a Spinco L-2 ultracentrifuge with a SW-50.1 rotor. Linear 5 to

20% sucrose gradients were made up in Tris-EDTA buffer without or with 0.5 M KCl. Polyacrylamide gel electrophoresis was carried out according to Davis (18) and Jarabak et al. (19) in pH 8.7 at 20°C. Isoelectric focusing was performed with an LKB 8101 apparatus with double-cooling jackets, in 2% ampholine in a 5 to 50% sucrose gradient as stabilizer. After a constant milliamperage was reached, 2-ml fractions were collected and pH and radioactivity were measured from each fraction. Radioactivity was determined in disposable polyethylene vials containing 10 ml of Insta-Gel (Packard Instrument Co., Ill., U.S.A.) using a Packard Tri-Carb liquid scintillation spectrometer.

Protein was estimated with the method of Lowry et al. (20) or with UV absorption at 280 nm. Alcohol dehydrogenase activity was assayed essentially as described by Racker (21).

Separation of bound and unbound radioactivity. In steroid-binding activity assays two methods for the separation of bound and unbound radioactivity were used: filtration over Sephadex G-25 (Tris-EDTA buffer, column dimensions 10 x 250 mm) or adsorption of the unbound radioactivity with dextran-coated charcoal (22). These two methods gave parallel results and the latter was chosen as a standard procedure on account of its convenience.

Table I. Partial purification of progesterone-binding protein from pregnant guinea pig uterus

Fraction	Total protein (mg)	Total radioactivity (cpm x 10^{-3})	Specific activity (cpm x 10^{-3}/ mg prot.)	Yield (%)	Purification (-fold)
1. 105,000 x g supernatant	1 110	9 825	8.8	100	1
2. First hydroxylapatite	5.25	4 611	878	47	99
3. Second hydroxylapatite	2.15	2 998	1393	31	157
4. Sephadex G-200	1.81	2 756	1524	28	172

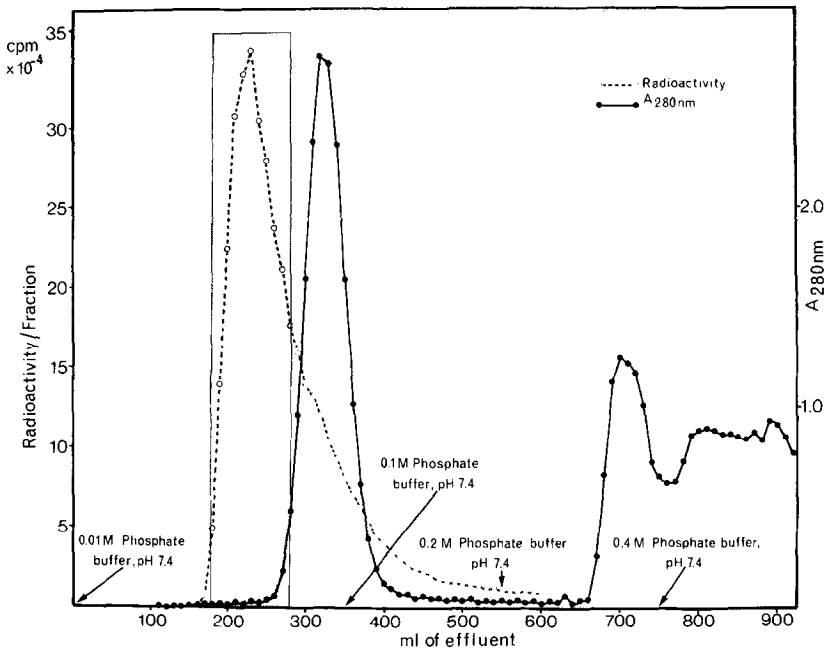

Figure 1. Purification of the 105,000 x g supernatant fraction (50 ml) from guinea pig uterus by chromatography on a hydroxylapatite column (30 x 420 mm). Prior to chromatography, the cytosol fraction was incubated 12 hours at +4°C with ^{3}H-labeled progesterone (3 x 10^{-9} mole/l). The column was eluted stepwise with 0.01, 0.1, 0.2 and 0.4 molar potassium phosphate buffer, pH 7.4, as indicated in the figure. The darkened area denotes fractions pooled for further purification steps.

RESULTS

Purification of the binding protein. Table I summarizes the purification procedure used. As seen in Fig. 1, a progesterone-macromolecule complex from guinea pig uterine cytosol was not adsorbed on the hydroxylapatite column, but was eluted shortly after the void volume. On the other hand, the bulk of cytosolic proteins were retained in the column and eluted with potassium phosphate buffer of higher molarity (Fig. 1). The two successive hydroxylapatite chromatographies resulted in a high degree of purification of the binding protein (Table 1).

The elution of the progesterone-binding protein on Sephadex G-200 is presented in Fig. 2. No significant additional purification was achieved with DEAE-cellulose. As seen in Table I, the binding protein was purified about 200-fold as compared with the crude cytosolic fraction.

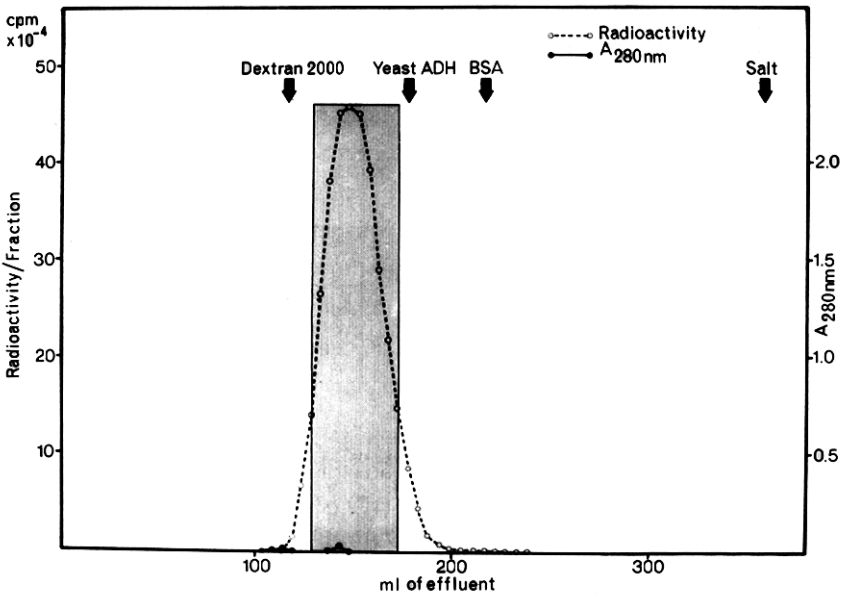

Figure 2 . Gel filtration on Sephadex G-200 (26 x 640 mm) of the progesterone-binding protein after the second chromatography on hydroxylapatite. Before the gel filtration the hydroxylapatite eluate was concentrated to a volume of 8 ml by ultrafiltration. The darkened area represents fractions pooled for further studies.

Nature of the partially purified protein. Molecular Stokes radius, as determined by the method of Ackers and Steere (17), was 5.5 nm for the progesterone-binding macromolecule. In the sucrose density gradient centrifugation, a single peak of bound radioactivity with a sedimentation

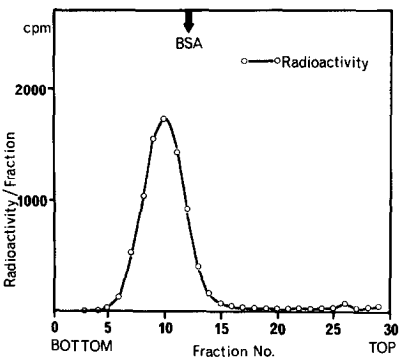

Figure 3. Sucrose gradient analysis of the progesterone-binding protein from guinea pig uterus. Sephadex G-200 eluate (0.2 ml) was layered over 5 to 20% sucrose gradient in Tris-EDTA buffer. Centrifugation was carried out in a Spinco SW-50.1 rotor at 45 000 rev./min for 14 hours at +2°C. 0.2-ml fractions were collected and counted for radioactivity.

coefficient of about 5 S was seen (Fig. 3). The apparent molecular weight of the protein was 106,000 when calculated on the basis of the data obtained from the gel filtration and the sucrose density gradient centrifugation experiments (17). The partial specific volume of the protein was assumed to be 0.73 ml/g. The molecular parameters did not undergo changes when gel filtration and density gradient centrifugation were performed in the buffer containing 0.5 M KCl. The presence of protein in the steroid-binding site was demonstrated by the destruction of the progesterone-macromolecule complex by incubation with Pronase (Sigma Co., 25 μg per incubation at 37°C for 2 hours). Similar treatments with ribonuclease, deoxyribonuclease and neuraminidase did not destroy the progesterone-macromolecule complex.

On polyacrylamide gel electrophoresis the progesterone-binding protein formed a single peak and had a relative mobility value of 0.21 (relative to bromphenol blue). Of the four bands visible after Amido black staining of the gel, the one migrating most slowly had the same relative mobility as the radioactive progesterone-protein complex. The purity of the progesterone-binding protein after gel filtration on Sephadex G-200 was calculated to be about 5 %. The isoelectric focusing experiments revealed the presence of one progesterone-binding component with an isoelectric point of about 2.5.

Table II summarizes the results obtained in studies on the affinity

Table II. Relative affinity[+] of the partially purified binding protein for different steroid ligands (progesterone = 1.00)

Steroid	Relative affinity
5α-Pregnane-3,20-dione	0.93
5β-Pregnane-3,20-dione	0.24
Pregnenolone	0.013
20α-Hydroxy-4-pregnen-3-one	0.016
20β-Hydroxy-4-pregnen-3-one	0.19
17α-Hydroxyprogesterone	0.019
Deoxycorticosterone	0.46
Cortisol	0.001
Testosterone	0.059
5α-Dihydrotestosterone	0.16
Estradiol-17β	0

[+] Relative affinity calculated as described in Ref. 25.

of the purified binding protein for different ligands. As seen in this table, the highest affinity was found for progesterone, followed by 5α-pregnane-3,20-dione and deoxycorticosterone. The partially purified protein showed practically no binding affinity for 17α-hydroxyprogesterone, cortisol or estradiol-17β. The association constant for progesterone was 3.2×10^9 1/mole at $+4^{\circ}C$, as measured from a Scatchard-type plot (23).

DISCUSSION

The presence of a specific high affinity progesterone-binding protein in the uterine cytosol of late-pregnant guinea pigs is demonstrated in this study.

This progesterone-binding protein seems to differ from that found in uteri of estrogen-treated animals (9,10). The receptor protein in uterine cytosol during pregnancy had a sedimentation coefficient of approximately 5 S in 10 mM Tris-EDTA buffer, whereas that obtained after estrogen priming was detected as a 6.7 S component. Furthermore, the former protein did not dissociate into subunits at higher ionic concentrations, in contrast to the latter which displayed a peak at 4.3 S when centrifuged through a sucrose gradient containing 0.3 M KCl (9). It seems that these differences are not due to more extensive purification of the binding protein in the pregnant uterus, because only a single peak of bound radioactivity at 5 S was found, when a crude uterine cytosol fraction labeled with ^{3}H-progesterone was centrifuged through a sucrose gradient with or without 0.5 M KCl.

In the course of pregnancy, the progesterone-binding capacity of guinea pig plasma increases about 100-fold as compared with that of non-pregnant animals (13,14). The possibility of contamination by plasma binding proteins can be ruled out for several reasons. Firstly, the binding protein could not be extracted from uterine tissue by several successive extensive washings with a large volume of the homogenization buffer. No hemoglobin could be detected in the uterine cytosol preparation after the rinsings. Secondly, the binding ability of uterine cytosol greatly exceeded that found in striated muscle, even though the hemoglobin content of the latter preparation was much higher. Thirdly, the molecular characteristics on sucrose gradient ultracentrifugation, disc gel electrophoresis, isoelectric focusing and gel filtration on Sephadex G-200, of the progesterone-binding protein in pregnant guinea pig blood plasma have been found to differ from those reported in this paper for progesterone-binding protein in pregnant guinea pig uterus (9,24).

The ligand specificity for the partially purified progesterone-binding protein from pregnant guinea pig uterus was comparable with that found by Wiest and Rao for binding proteins from human endometrium and rabbit uterine tissues (10).

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