Journal of Steroid Biochemistry, 1977, Vol. 8, pp. 1179–1182. Pergamon Press. Printed in Great Britain

SEPARATION OF PROGESTERONE-SPECIFIC FROM CBG-LIKE BINDING SITES BY CHROMATOGRAPHY ON COLUMNS OF SPHEROIDAL HYDROXYLAPATITE*

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(*Received* 24 *February* 1977)

SUMMARY

Columns of spheroidal hydroxylapatite were used to separate progesterone-specific from CBG-like binding sites in preparations of human or rat uterine cytosol. The advantages of the method are a rapid and complete separation of the two types of progesterone-binding proteins with good recoveries. Up to a 10-fold purification of the progesterone-specific binding protein may be achieved.

INTRODUCTION

Most uterine cytosol preparations contain at least two high affinity progesterone-binding proteins, a progesterone-specific binding protein (*R*), the putative progesterone receptor, and a binding protein (*G*) resembling corticosteroid-binding globulin (CBG-like) [1–4]. Investigation of the properties of the progesterone-specific protein have been hampered by the presence of large amounts of CBG-like binding in cytosol preparations.

Some investigators [4–6] have used ammonium sulfate precipitation to separate the two types of sites, but when applied to rat uterine cytosols, this procedure did not completely separate the binding sites [1]. A batch procedure using conventional hydroxylapatite also failed to give complete separation [1] and flow rates were too slow using columns of this material. This paper describes the preparation of columns of spheroidal hydroxylapatite and their use in the separation of the two types of binding sites present in rat or human uterine cytosol preparations.

EXPERIMENTAL

Radioactive steroids, [1,2,6,7-^{3}H]-progesterone (S.A. = 103.7 Ci/mmol), [1,2-^{3}H]-progesterone and [1,2-^{3}H]-cortisol (S.A. $\simeq$ 50 Ci/mmol), were obtained from New England Nuclear Corporation (Boston, MA); purity was checked by t.l.c. Preparations less than 97% pure or with a single impurity of 2% or more of the radioactivity were purified by t.l.c. Unlabeled progesterone from Schwarz/Mann (Orangeburg, N.Y.) and cortisol from Sigma Chemical Company (St. Louis, MO) were used without further purification.

The preparation of rat uterine cytosol in TEG buffer (10 mM Tris, 1 mM Na_2EDTA, 20% glycerol, pH 7.4) and the DEAE-cellulose filtration assay used to measure the capacities of the two types of binding sites have been described [1, 2]. Human uterine cytosol was prepared as previously described [3] with the inclusion of 5 mM dithiothreitol in the buffer (TEGD). A Polytron PT-10 (Brinkmann Instruments, Switzerland) was used to homogenize rat and human uteri [7]. All work was done at 4°C.

Spheroidal hydroxylapatite (BDH, Gallard-Schlesinger, Carle Place, NY) was prepared for use according to the manufacturer's instructions (BDH Chemicals, Poole, England). It was washed first with 0.2 M NaOH, then with water, and the fine particles were decanted. Columns of spheroidal hydroxylapatite in TEG or TEGD were poured in glass syringes which were plugged at the bottom with glass wool.

Cytosol was applied to a column and fraction collection began as the cytosol entered the hydroxylapatite. A stepwise increase in phosphate concentration was used for elution. Although the general method was the same, there were minor differences in the procedures depending on whether rat or human uterine preparations were used. The details of individual separations are described in the figure legends.

It takes about 1 h to complete a fractionation. After use the columns were washed with 0.4 M phosphate followed by 0.1 M NaOH and were re-equilibrated with the starting buffer before the next run.

The column fractions were dialyzed against TEG or TEGD for 3 h with one change of buffer to remove excess phosphate. After dialysis the fractions were analyzed for steroid binding using the DEAE-cellulose filtration assay [1].

* Portions of this work are from a thesis submitted by B. A. Booth to the University of Wisconsin in partial fulfillment of the requirements for the Ph.D. degree.

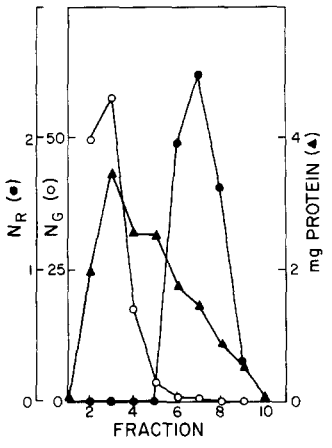

Fig. 1. Separation of *R* from *G* sites by chromatography of rat uterine cytosol on 2 g spheroidal hydroxylapatite. The column (0.85 × 4.3 cm) was equilibrated with TEG and loaded with 2 ml of cytosol in TEG. Fraction collection (1.5 ml) was then begun. After the sample ran into the hydroxylapatite, 2 ml of 0.004 M NaPG was added followed by 2 ml of 0.08 M, 2 ml of 0.16 M, and 4 ml of 0.32 M NaPG. The column was washed with about 4 ml of 0.4 M NaP and then with about 2 ml of 0.1 M NaOH and re-equilibrated with TEG. Fractions 2–9 were dialyzed against TEG as described in the text. The filtration assay [1] was used to determine N_R and N_G which are expressed as pmol sites/fraction. Of the 15.4 mg of protein loaded on the column, 15.3 mg (99%) was recovered.

Some human uterine cytosol preparations were fractionated with $(NH_4)_2SO_4$ prior to hydroxylapatite chromatography. Saturated $(NH_4)_2SO_4$ in TEGD was added to the cytosol dropwise with gentle mixing until 30% saturation was reached. After 30 min the preparation was centrifuged at 2000 g for 30 min. The precipitation was adequate only in cytosol prepared without glycerol. The supernatant was removed and the pellet was resuspended in TEGD and chromatographed on spheroidal hydroxylapatite.

Protein concentrations were determined by the method of Lowry *et al.*[8] using bovine serum albumin as the protein standard. Dithiothreitol was removed prior to protein determination by dialysis against excess TEG.

RESULTS AND DISCUSSION

The CBG-like binding protein is not adsorbed by hydroxylapatite while the progesterone-specific binding protein is [1]. Figure 1 shows the elution patterns of the two types of binding sites in rat uterine cytosol from a hydroxylapatite column. For the *G* sites the elution volume was the void volume (fractions 2 and 3) while the progesterone-specific sites were eluted with 0.16 M phosphate (fractions 6–8) and called the *R* pool. Application of up to 7 mg of cytosol protein/g of hydroxylapatite did not result in an altered protein elution profile. Protein recovery in six fractionations of rat uterine cytosol was 96.0 ± 4.3% (mean ± S.D.). In one experiment, endogenous progesterone concentrations were measured in the cytosol and in the *R*-pool by RIA (9); 94% of the progesterone was removed by the chromatography and dialysis.

When cytosol prepared from intact rats is fractionated it is difficult to estimate the recovery of binding sites because of interference by endogenous steroids in the cytosol. In order to estimate recovery of *R* and *G* sites, cytosol was prepared from the uteri of adrenalectomized–ovariectomized rats. There were 23.1 ± 1.9 pmol (±S.D.) of *R* sites in the sample of unfractionated cytosol placed on the column. Of these sites, 1.0 ± 0.6 pmol were recovered in fractions 2–3, 2.8 ± 1.3 pmol in fractions 4–5, and 19.2 ± 2.9 pmol in fractions 6–8 (the *R* pool). The total recovery was, therefore, 99.6% with 83% of the *R* sites present in the *R* pool. Similarly, there were 136 ± 27 pmol of *G* sites loaded on the column. Of these sites, 122 ± 2.9 pmol were recovered in fractions 2–3 and 17 ± 4.4 pmol in fractions 4–5 for a recovery of 102%. No *G* sites were detected in fractions 6–8.

Figure 2 shows hydroxylapatite elution patterns of radioactivity and protein from a fractionation of

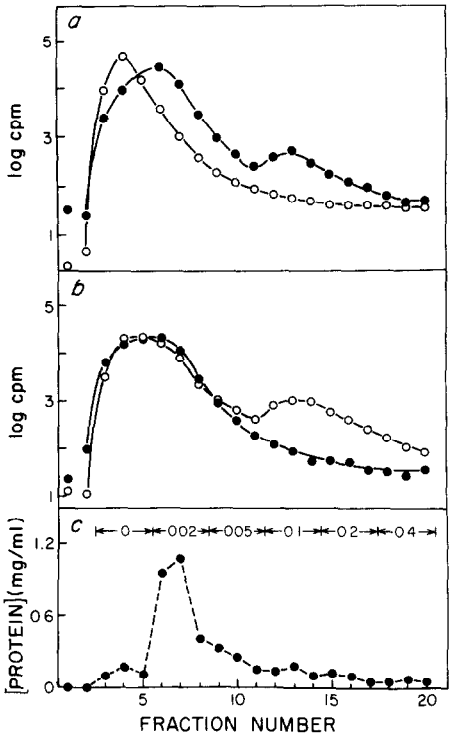

Fig. 2. Elution profiles of [1,2,6,7-^{3}H]-progesterone and human myometrial cytosol from spheroidal hydroxylapatite. The sample and fraction vols were 5 ml; duplicate 100 μl samples of each fraction were counted. The molarity of phosphate in TEGD used for the elution is indicated between arrows at the top of panel c. The column, prepared from 10 g hydroxylapatite in a 20 ml syringe, had a void volume (fractions 1 and 2) of 10 ml. (a) Elution of 10 nM [^{3}H]-progesterone alone (○) and incubated with cytosol (●). (b) Elution of cytosol incubated with 10 nM [^{3}H]-progesterone plus 2.5 μM cortisol (○) or progesterone (●). (c) Elution profile of protein (21.5 mg applied, 102% recovery).

Table 1. Separability of the *R* and *G* sites present in human and rat uterine cytosols fractionated by spheroidal hydroxylapatite columns

Sites and preparations	Human	Rat
R sites in *G*-pool	3.5 ± 1.2 (5)	−2.1 ± 2.7 (5)*
G sites in *R*-pool	1.8 ± 1.3 (5)	6.3 ± 4.8 (14)
G sites in cytosol, before fractionation†	65.5 ± 4.5 (5)	95.5 ± 1.5 (8)

For each type of sites, the mean concentration is given as a percentage of the total concentration of high-affinity binding sites for progesterone (*R* + *G*) present in the preparation. The S.E.M. and the number of fractionations (in parentheses) are also given.

* Not significantly different from zero. † The *R* sites equal 100-*G* sites, with the same S.E.M.

human uterine cytosol (4.3 mg protein/ml). Protein recovery in six fractionations of human uterine cytosol was 99.2 ± 2.7% (mean ± S.D.). When [^{3}H]-progesterone was chromatographed alone, there was one peak or radioactivity at the void volume of the column (Fig. 2a). Chromatography of human uterine cytosol preincubated with [^{3}H]-progesterone resulted in a broader peak at the void volume and the appearance of a second peak of radioactivity eluting with 0.1 M phosphate. The broadened initial peak was a composite of the free and *G*-bound steroid, eluted by the phosphate-free buffer, and the non-specific binding to proteins in the 0.02 M phosphate fractions (fractions 6–8) which contained the majority of the protein (Fig 2c). The second peak of radioactivity, which eluted with the fractions expected to contain the *R* sites (*R* pool, fractions 12–14), was still present when the cytosol-steroid incubation included a 250-fold molar excess of cortisol but was eliminated by inclusion of a 250-fold excess of progesterone in the incubation (Fig. 2b). Most of the [^{3}H]-progesterone was removed from the fractions containing the second peak of radioactivity by dialysis. When the *R* pool was filtered through DEAE-cellulose discs before dialysis, about 25% of the [^{3}H]-progesterone was present in bound form. We infer from this that there was partial, but not complete, dissociation of progesterone-*R* complexes during the elution process.

In all chromatographies of human uterine cytosol, *R* sites were eluted when the phosphate concentration was at least 0.1 M, but the *G* sites were eluted in phosphate-free buffer, resulting in elution profiles similar to those using rat uterine preparations. Human *R* pool labeled with 2 nM [^{3}H]-progesterone in TEGD showed no significant loss of binding activity for up to 16 days when kept on dry ice.

We confirmed the absence of CBG-like contamination in the *R* pool in two ways: (1) There was no significant amount of cortisol bound by the *R* pool (Table 1); (2) The percentage of progesterone bound in the presence and absence of excess unlabeled cortisol remained unchanged in the *R* pool from human or rat cytosols.

Most of the uterine cytosol protein was eluted from the spheroidal hydroxylapatite by low phosphate concentrations (Figs 1, 2c) and up to a 10-fold purification of the *R* sites was achieved. When human uterine cytosol was brought to 30% saturation with $(NH_4)_2SO_4$, the precipitate contained all of the *R* sites (presumably in the activated form), some of the *G* sites, and 25–30% of the protein. Hydroxylapatite chromatography of this fraction yielded an *R* pool which contained only 1–2% of the total cytosol protein, but the recovery of *R* sites was poorer, suggesting that under these conditions the *R* sites are less stable.

Hydroxylapatite has been used to purify plasma CBG [10] and in the partial purification of progesterone-binding proteins [11]. It has also been used to assay estrogen receptors [12, 13]. Fractionation of human myometrial cytosol on conventional hydroxylapatite [14] led others to conclude that only CBG-like binding was present. Chromatography on columns of spheroidal hydroxylapatite represents a significant improvement because it provides a rapid and complete separation of the two types of high-affinity binding proteins while removing almost all of the endogenous steroid. This separation is an important step in the study of progesterone action on the mammalian uterus. Our previous reports of studies on uterine progesterone-binding proteins [1–3] discussed some of the difficulties in establishing statistically reliable binding parameters for either protein when both are present. In addition, rapid and complete separation of the two types of binding proteins will be advantageous in studies of steroid–nucleus interactions.

Acknowledgements—We are grateful to P. L. Miller for assistance in preparing and typing the manuscript. The Endocrinology-Reproductive Physiology Program provided traineeships for B. A. Booth and R. D. Dyer. The work was supported in part by PHS Training Grant No. 5-T01-HD-00104-10 from NICHHD, Ford Foundation Grant No. 630-0505B,C, and University of Wisconsin Graduate School funds.

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