Journal of Chromatography, 397 (1987) 269–278
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1222

PROGESTIN RECEPTORS FROM TISSUES EITHER EXHIBITING OR LACKING ESTROGEN RESPONSE MECHANISMS

COMPARISON OF CONVENTIONAL AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHODOLOGY

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SUMMARY

Evidence from a variety of target organs has shown that progesterone receptor (PR) is induced by estrogen receptor (ER) in normal and neoplastic tissues. However, approximately 12%of the normal human uterine samples exhibit only PR with no measurable ER, suggesting the expression of both inducible and constitutive receptor isoforms. We investigated several molecular properties of PR from tissues either exhibiting or lacking ER. All studies were conducted in potassium phosphate buffer containing 10 m*M* sodium molybdate with a synthetic progestin, [^{3}H]R5020 as the ligand. Radioinert R5020 was used as competitor to assess nonspecific association. Competition analysis showed that PR from both sources exhibited similar ligand specificities and affinities. Relative affinities were ORG 2058 > R5020 > medroxyprogesterone acetate > progesterone ≫ testosterone (K_d values ranged from 10^{-9} to 10^{-10} *M*; testosterone showed no specific competition). We utilized high-performance liquid chromatography in the size-exclusion (HPSEC) and ion-exchange (HPIEC) modes to probe the size and ionic properties of PR. HPSEC profiles showed that the PR isoform from both sources was eluted as a single, sharp peak >75 Å. HPIEC elution profiles indicated no differences in the surface ionic properties in that PR from both tissue types eluted with *ca.* 100 m*M* phosphate. These experiments show no difference between the inducible and the putative constitutive form of PR. Thus, some PR species may not require estrogen for their formation.

INTRODUCTION

Biochemical characterization of steroid hormone receptors and their regulation are important diagnostic indicators of the course of neoplastic disease involving steroid-sensitive tissues, such as the breast and uterus[1–3]. Although our knowledge and the clinical use of estrogen receptor (ER) measurements are greater than those of progesterone receptors (PR), it appears that PR will play an increasingly important role in the therapeutic rationale for patients with steroid-sensitive neoplasia[2–4].

0021-9673/87/$03.50

The clinical importance of PR in humans was recognized with the findings that both in rodents *in vivo* and in human breast cancer cells (T47D), the PR is under the regulation of ER[5,6]. After complexing with estrogens, the ER interacts with the nucleus and induces PR in a time-dependent fashion. The induction of PR is also cycloheximide-sensitive, indicating synthesis of new protein rather than simply protein stabilization. Therefore, the measurement of PR is important in identifying endocrine-responsive patients, since its presence indicates an intact endocrine mechanism. This will avoid any delay in chemotherapy of patients with tumors insensitive to endocrine manipulation[2,3], such as treatment with tamoxifen.

The structural properties of PR have best been studied in chick oviduct and human breast cancer cells[6–8]. Two subunits of PR have been identified: subunit A of 81 kilodalton with high affinity for DNA and subunit B of 115 kilodalton with high affinity for chromatin. These subunits have been shown to be regulated independently[8] and control PR sensitivity in the chick oviduct. In an extensive series of studies in our laboratory, we hasve found that *ca.* 12% of normal human uterine samples exhibit PR but no measurable ER[4]. This situation is unique, since it indicates the possibility of two types of PR, *i.e.* inducible and constitutive isoforms.

In this report, we have explored further several physical and kinetic properties of the PR from tissues either containing or lacking ER in order to ascertain whether these represent two different species of PR. We utilized sodium molybdate in all assay buffers, since it has been shown to stabilize the PR on extraction in its non-activated form[9]. To study the kinetic properties, we utilized synthetic progesterone analogues and compared the specificity and affinity displayed by PR in the two types of receptor preparations, *i.e.* ER positive and negative. For the chromatographic comparison, high-performance liquid chromatography (HPLC) in the size-exclusion (HPSEC) and ion-exchange (HPIEC) modes was employed. The two techniques are extensively used in our laboratory for the separation and characterization of steroid receptor isoforms[10–12].

EXPERIMENTAL

Tissue collection

Residual tissue from human uteri was obtained following hysterectomy for a variety of clinical conditions through the cooperation of the Pathology Departments at Norton Kosairs Childrens Hospital and at Humana Hospital University (Louisville, KY, U.S.A.), These tissues were weighed and immediately placed on ice and transported to the laboratory. Samples were taken for histopathology, and the remainder was stored at −90°C until utilized for studies reported in this paper. A pilot study conducted in our clinical laboratory determined the status of ER and PR in these tissues by the titration assay[1].

Cytosol preparations

All tissue processing was accomplished at 4°C. Tissues were homogenized in phosphate buffer (10 m*M* potassium dihydrogenphosphate–1.5 m*M* EDTA–1 m*M* dithiothreitol–10% (v/v) glycerol–10 m*M* sodium molybdate, pH 7.4 at 4°C), $P_{10}M$, by means of a Brinkman Polytron, delivering two 10-s bursts. Cytosols were prepared by centrifugation of the tissue homogenates for 30 min at 105000 *g* followed by

removal of the lower layer. A protein concentration of *ca.* 2 mg/ml was achieved. Cytosol preparations were diluted if necessary with $P_{10}M$ to adjust the protein concentration to *ca.* 2 mg/ml. Such a preparation was utilized in Scatchard[13] and competition analyses. All HPLC analyses were performed on cytosols prepared by homogenizing *ca.* 300–400 mg of tissue in 1 ml of $P_{10}M$. The approximate protein concentration was 10 mg/ml.

Protein determination

Protein was determined by the method of Bradford[14] with reagents obtained from Bio-Rad (Richmond, CA, U.S.A.) using bovine serum albumin (BSA) as standard. Protein standards were prepared in molybdate-containing buffers, and used to construct the standard curve.

Scatchard analysis

Cytosol (100 μl) prepared as described above, was incubated with six different concentrations of [^{3}H]R5020 (range 0.15–5.0 n*M*) in the presence and absence of radioinert R5020 (1.0 μ*M*) for 16–24 h. Both types of R5020 were obtained from DuPont/NEN Products (Boston, MA, U.S.A.). After the addition of 300 μl of 5% dextran-coated charcoal (DCC) to the reaction mixture, it was Vortex-mixed, then incubated for 15 min, and finally centrifuged at 1500 *g* for 15 min. The supernatants were decanted into glass counting vials, and 4 ml of a toluene Triton-based scintillation cocktail was added; the vials were shaken and counted for 5 min.

Competition analysis

Cytosol (100 μl) was incubated with varying concentrations of radioinert R5020 (NEN/DuPont), ORG-2058, medroxyprogesterone acetate (MPA/Amersham, Arlington Heights, IN, U.S.A.), progesterone and testosterone (Sigma, St. Louis, MO, U.S.A.) in the presence of a single concentration (5 n*M*) of [^{3}H]R5020. The concentrations of radioinert competitors to [^{3}H]R5020 were chosen to give ratios in the range of 1:10 to 3000:1. The reaction mixture was incubated for 16–24 h and then the free steroids were removed with DCC, as described in the previous section. An aliquot was analyzed for radioisotope detection. To calculate the K_d (dissociation constant) value of the radiolabeled ligand, a separate titration and Scatchard analysis on the cytosol preparation used for competition analysis[15] was performed at the same time.

HPLC of PR

Chromatography was always performed at 0–4°C. All buffers were filtered through a 0.45-μm Millipore (Bedford, MA, U.S.A.) filter. Cytosols were allowed to react with 5 n*M* [^{3}H]R5020 in the absence or presence of a 200-fold excess of radioinert R5020 for 3–7 h. The reaction mixture was then cleared of unbound ligand with 1% DCC for 5 min and centrifuged at 1500 *g* for 5 min. The supernatant was decanted and aliquots (300 μl each) were injected simultaneously into both ion-exchange and size-exclusion columns (see below). A separate aliquot was taken to determine the injected radioactivity. In our analysis, recoveries from both of the columns (ion-exchange and size-exclusion) ranged from 75 to 100%.

HPIEC

The DCC-treated reaction mixture (300 μl) was applied to a silica based AX-1000 (SynChrom, Lafayette, IN, U.S.A.) anion-exchange column, 25 × 4.1 cm, previously equilibrated with $P_{10}M$. Chromatography was performed at a flow-rate of 1 ml/min, using a Beckman (Fullerton, CA, U.S.A.) Model 114 two-pump solvent delivery system. Following sample application, the column was washed for 10 min with $P_{10}M$. This was followed by elution of protein (radioactivity) with a linear gradient of phosphate approaching 300 m*M* after 49 min. A linear gradient was obtained by means of pump B by mixing $P_{500}M$ (500 m*M* phosphate + all other components in $P_{10}M$) with $P_{10}M$ with a predetermined program in the system controller (Beckman Model 421). Fractions (1 ml) were collected and their phosphate concentration was determined by measuring the conductivity relative to standards. Aliquots (200 μl) were also taken for radioactivity determinations.

HPSEC

This mode of chromatography was performed isocratically[11,16]. The reaction mixture (300 μl) was applied to a TSK-3000 SW (Toyo/Soda, Japan) size exclusion column (60 × 0.7 cm), equilibrated with $P_{50}M$ (50 m*M* phosphate + all other components in $P_{10}M$). Elution was performed at 0.7 ml/min, and 0.5-min fractions were collected. Radioactivity in each fraction was determined as previously described. Calibration of the column was performed with standard protein solutions of cytochrome *c* (Cc), bovine serum albumin (BSA), catalase (Cat), ferritin (Fe) and thyroglobulin (T). The void volume (V_0) was determined with Blue Dextran.

RESULT AND DISCUSSION

It is now known from a multitude of analyses carried out in our laboratory[1,4,17] and others[2,3,5] that a certain proportion of both human breast and uterine cancer specimens exhibit PR in the absence of ER. This is intriguing, since it appears that PR is under the control of ER[5–8]. Nevertheless, the polymorphism which seems to be associated with nearly all of the steroid hormone receptors analyzed thus far indicates the possibility of a tissue (clone of cells) possessing subunit(s) of PR which are constitutively produced and therefore may *not* represent a marker of estrogen action.

In the present investigation, we analyzed PR in human uterine tissues which either lacked ER (abnormal condition) or possessed ER (normal condition). This was undertaken by analyzing certain PR kinetic and structural properties in the two tissue sources with the possibility of differentiating those receptor molecules which were under ER regulatory mechanisms from those which were independent of ER action. The structural aspects of the study were performed by employing the sensitive procedure of HPLC, which was already well established in our laboratory, for studying steroid receptor[12]. One advantage in establishing a comparison was the fact that we could use sodium molybdate, a receptor-stabilizing agent[9], in both homogenization and assay buffers as well as in all the mobile phases used for HPLC analysis.

Fig. 1 demonstrates the distribution of PR concentration in human uterine biopsy samples from 394 patients. The solid bars represent receptor concentrations in those samples which lacked ER (ER^-). Generally, these appeared at the lower

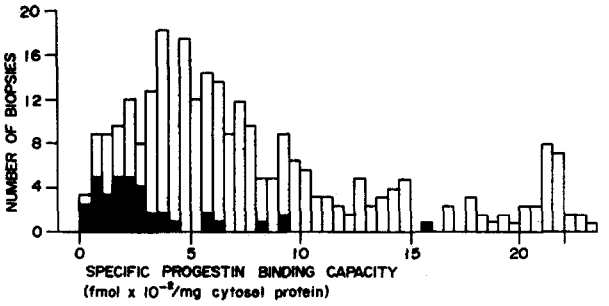

Fig. 1. Distribution of progestin receptors from the ER^+ and ER^- human uterine biopsy specimens. (□) ER^+/PR^+ (n = 347); (■) ER^-/PR^+ (n = 47).

range of PR content. The influence of ER concentration on the presence of PR is not well understood in human uterus. Clearly the concentration of ER may not be the only parameter responsible for the induction of PR as has been observed in breast cancer cells[5,6]. The absence of PR (PR^-) may result from a defect at the genetic level which would normally respond to ER. Results with T47-D breast cancer cells showing high PR levels in the absence of ER support the idea of a constitutive synthesis of certain PR[5,6]. Our data show no significant difference in the appearance of the two types of tissues under investigation. The distribution of the two types of receptor populations, *i.e.* ER^-/PR^+ and ER^+/PR^+, in human uteri was similar to that observed in human breast cancer[18].

Fig. 2 substantiates this result since there was no significant difference observed in the values of the dissociation constants (K_d) of the two different types of human uterine biopsies. These data indicate that the difference, if any, in the molecular parameters of the native and putative constitutive isoforms of these receptors seems unlikely to reside in these kinetic parameters.

Figs. 3 and 4 provide representative titration curves of PR in tissues exhibiting and lacking ER, respectively. The insets show Scatchard analysis data for PR which were typically obtained from tissues either containing ER (Fig. 3) or lacking ER (Fig. 4), the PR-regulatory protein. Although there was a difference in the specific binding

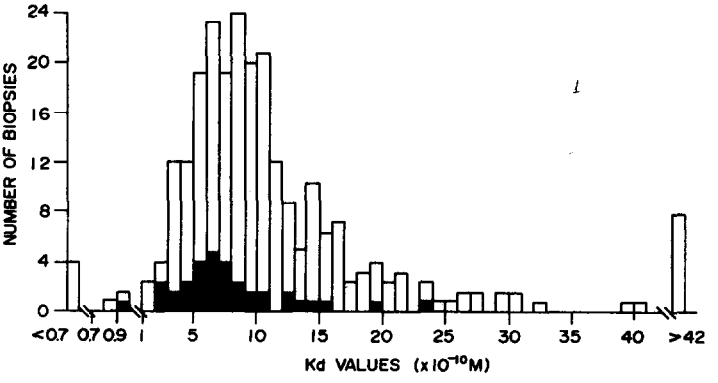

Fig. 2. Distribution of dissociation constant (K_d) values for progestin receptors from ER^+ and ER^- human uterine biopsy specimens. (□) ER^+/PR^+; (■) ER^-/PR^+.

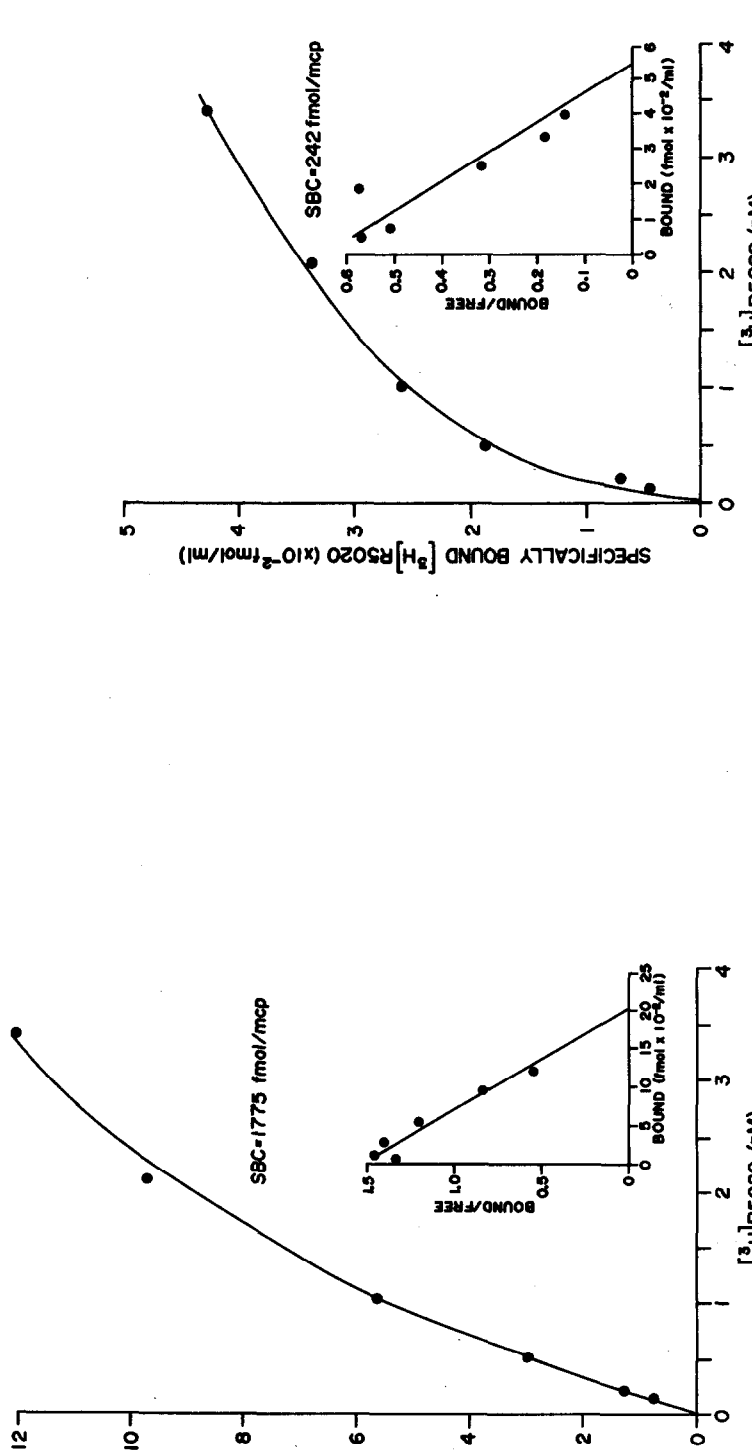

Fig. 3. Representative titration analysis of PR from a human uterine biopsy sample containing ER. Details are provided in Experimental. Data from specific binding curves were plotted according to the method of Scatchard (inset) and the dissociation constant (K_d) was determined from the slope of this plot.

Fig. 4. Representative titration analysis of PR from a human uterine biopsy sample lacking ER. Details are provided in Experimental. Data from specific binding curves were plotted according to the method of Scatchard (inset) and the dissociation constant (K_d) was determined from the slope of this plot.

TABLE I

LIGAND SPECIFICITY OF PROGESTIN RECEPTORS IN UTERI WITH AND WITHOUT ESTROGEN RECEPTORS

$$K_c \simeq K_s \frac{[I_{50}\text{ (competitor)}]}{[I_{50}\text{ (R5020)}]}$$

I_{50} = concentration to produce 50% inhibition of [^{3}H]R5020 binding, K_c = apparent dissociation constant of competitor, K_s = apparent dissociation constant of [^{3}H]R5020 as determined by Scatchard analysis.

*Receptor status of tissue**	*K_d of competitor (nM)*			
	ORG-2058	*MPA*	*Progesterone*	*Testosterone*
ER^+/PR^+	0.4	1.5	1.3	UD**
ER^+/PR^+	0.8	2.4	2.0	UD
ER^-/PR^+	0.6	1.1	1.5	UD
ER^-/PR^+	0.6	1.1	2.7	UD

* Values shown are for four different uteri.
** UD = undetectable.

capacities of these two representative uteri, these data did not show any difference in the K_d values. Both types of tissues exhibited K_d values that ranged from 10^{-9} to 10^{-10} M.

We further probed the properties of PR from the two tissues types using the competition analysis (see Experimental) with various synthetic ligands, mainly progestins and testosterone. Results shown in Tables I and II indicate no apparent difference in the competition by radioinert steroids for the ligand binding site of PR which was labeled with the synthetic ligand R5020. Thus, the ligand binding specificities were virtually identical, and the relative affinities of various steroids were in the order: ORG 2058 > R5020 > MPA > progesterone $\gg$ testosterone. Again,

TABLE II

LIGAND SPECIFICITY OF PROGESTIN RECEPTORS IN UTERI WITH AND WITHOUT ESTROGEN RECEPTORS

$$\text{Relative Affinity (RA)} = \frac{I_{50}\text{ of R5020}}{I_{50}\text{ of competitor}} \times 100\%$$

I_{50} = concentration to produce 50% inhibition of [^{3}H]R5020 binding.

*Receptor status of tissue**	*RA of competitor (% of control)*			
	ORG-2058	*MPA*	*Progesterone*	*Testosterone*
ER^+/PR^+	97	44	34	<1
ER^+/PR^+	130	49	53	<1
ER^-/PR^+	100	53	42	<1
ER^-/PR^+	69	45	15	<1

* Values shown are for four different uteri.

bassed on this sensitive technique, there appears to be no difference in PR isoforms from the two different types of human uterine tissues, suggesting that the basic structures of their ligand recognition sites are similar.

We have conducted HPLC analyses of steroid receptors in various modes[10–12,16,19]. Recently, using an in-line radioactivity detector (Beckman Model 170) and a Bio-Rad conductivity monitor we discovered that ER from lactating mammary glands exhibited receptor isoforms (polymorphism) which were dependent on their stage of differentiation[20]. This indicates that receptor structure, as reflected by isoform profiles from HPLC analyses may exhibit characteristics which are of biological significance. For this reason, we used HPLC to assess receptor structural differences in PR from the two different sources of human uterine tissues.

Fig. 5 presents representative HPSEC profiles of PR from tissues either exhibiting or lacking ER. PR from either source was eluted with similar retention times, representing a Stokes radius of *ca.* 75–85 Å. Also, the HPIEC profiles of PR isoforms from the two types of uteri (ER^+ and ER^-) showed similar patterns (Fig. 6) when eluted from an anion-exchange column (AX-1000). A single isoform eluting with *ca.* 100 m*M* phosphate was observed, suggesting that similar ionic charges were involved in the PR interaction with the column. This observation with respect to the elution pattern was consistent for both ER and PR isoforms from several tissue sources, suggesting a commonality in the steroid receptor structure which may have been preserved during evolution[21]. Analysis based on both HPSEC and HPIEC confirmed the similarity between PR from tissues exhibiting and lacking ER.

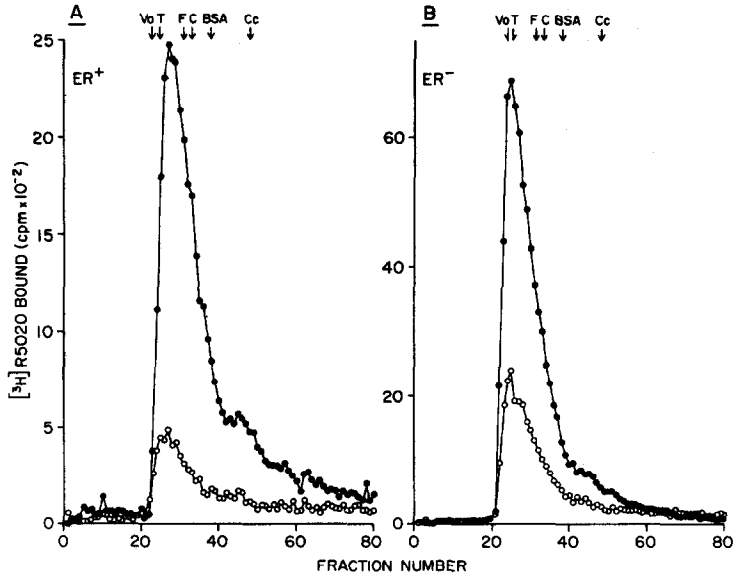

Fig. 5. Separation of human uterine PR by HPSEC. Cytosols from human uteri representing ER^+ (A) and ER^- (B) tissues were incubated with 5 n*M* [^{3}H]R5020 in the absence (●) and presence (○) of a 200-fold excess of unlabeled R5020 for 2 h at 4°C. Following removal of excess steroid with DCC, the samples were separated on TSK-3000 SW size-exclusion columns as described in Experimental.

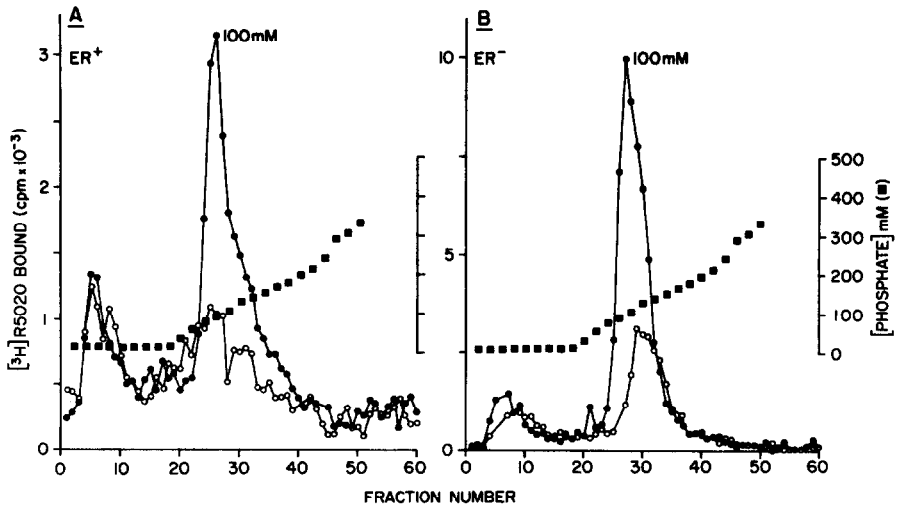

Fig. 6. Separation of human uterine PR by HPIEC. Cytosols from human uteri representing ER^+ (A) and ER^- (B) tissues were incubated with 5 n*M* [^{3}H]R5020 in the absence (●) and presence (○) of a 200-fold excess unlabeled R5020 for 2 h at 4°C. Following removal of excess steroid with DCC, the receptors were separated on an AX-1000 anion-exchange column with a linear gradient of phosphate buffer. Details are given in Experimental.

Thus far, we have found no significant difference between the characteristics of PR arising under normal regulatory conditions (ER^+/PR^+ tissues) from those PR which may represent constitutive synthesis (ER^-/PR^+ tissues). Presence of PR in an ER^- tissue may indicate that only a portion of the PR molecule (not the polypeptide containing the ligand binding domain) is under the influence of ER. Our recent discovery that certain isoforms of ER possess protein kinase activity[19,22] and the recent report that PR also possessed protein kinase activity[23] suggest more refined technology such as HPLC will be useful in the structural analysis of receptors. To expand this study, we are investigating the mechanisms of PR induction, realizing that the steroid binding domain of receptor molecules may represent only a portion of a far more complicated regulatory protein.

ACKNOWLEDGEMENTS

This research was supported in part by the Marie Overbey Memorial Grant from the American Cancer Society and by the Phi Beta Psi Sorority, F.P.K. was a medical student research fellow. S.M.H. is a research fellow of the Graduate School, University of Louisville. The authors appreciate the cooperation of Drs. Joseph Sanfilippo and Thomas C. Day of the Department of Obstetrics and Gynecology and Drs. James G. Kuhns and Stuart Wolfson of NKC Hospital.

REFERENCES

1 J. L. Wittliff, *Cancer*, 53 (1984) 630.
2 G. M. Clark, W. L. McGuire, C. A. Hubar, O. H. Pearson and J. S. Marshall, *New Engl. J. Med.*, 309 (1983) 1343.
3 W. T. Creasman, J. T. Soper, K. S. McCarty Jr., W. Hinshaw and D. L. Clarke-Pearson, *Am. J. Obstet. Gynecol.*, 151 (1985) 922.
4 L. A. van der Walt, J. S. Sanfilippo, J. E. Siegel and J. L. Wittliff, *Clin. Physiol. Biochem.*, 4 (1980) 217.
5 K. B. Horwitz, W. L. McGuire, O. H. Pearson and A. Segaloff, *Science*, 189 (1975) 726.
6 K. B. Horwitz and P. S. Alexander, *Endocrinol.*, 113 (1983) 2195.
7 W. T. Schrader and B. W. O'Malley, *J. Biol. Chem.*, 247 (1972) 51.
8 P. A. Boyd and T. C. Spelsberg, *Biochemistry*, 18 (1979) 3679.
9 T. J. Chen, R. G. MacDonald and W. W. Leavitt, *Biochemistry*, 30 (1981) 3405.
10 R. D. Wiehle and J. L. Wittliff, *J. Chromatogr.*, 297 (1984) 313.
11 R. D. Wiehle, G. E. Hofmann, A. Fuchs and J. L. Wittliff, *J. Chromatogr.*, 307 (1984) 39.
12 J. L. Wittliff, N. A. Shahabi, S. M. Hyder, L. A. van der Walt, L. Myatt, Y.-J. He and D. M. Boyle, in J. L'Italien (Editor), *Modern Methods In Protein Chemistry*, Plenum Press, NY, in press.
13 G. Scatchard, *Ann. N.Y. Acad. Sci.*, 51 (1949) 248.
14 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
15 D. Rodbard, in B. W. O'Malley and A. R. Means (Editors), *Receptors for Reproductive Hormones*, Plenum Press, NY, Vol. 36, 1973, p. 289.
16 L. A. van der Walt and J. L. Wittliff, *J. Steroid Biochem.*, 24 (1986) 377.
17 J. A. Carlson, J. C. Allegra, T. C. Day Jr. and J. L. Wittliff, *Am. J. Obstet. Gynecol.*, 149 (1984) 149.
18 Anonymous, *Cancer Suppl.*, 46 (1980) 2759.
19 S. M. Hyder, N. Sato and J. L. Wittliff, *J. Chromatogr.*, 397 (1987) 251.
20 S. M. Hyder, R. D. Wiehle and J. L. Wittliff, *Fed. Proc.*, 44 (1984) 1474.
21 J. L. Wittliff and R. D. Wiehle, in V. P. Holland (Editor), *Hormonally Respoonsive Tumors*, Academic Press, New York, Vol. 15, 1985, p. 383.
22 A. Baldi, D. Boyle and J. L. Wittliff, *Biochem. Biophys. Res. Commun.*, 135 (1986) 597.
23 T. Garcia, P. Tuohimaa, J. Mester, T. Buchou, J.-M. Renoir and E. E. Baulieu, *Biochem. Biophys. Res. Commun.*, 113 (1983) 960.