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HIGH-PERFORMANCE CHROMATOFOCUSING AND SIZE-EXCLUSION CHROMATOGRAPHY: SEPARATION OF HUMAN UTERINE ESTROGEN-BINDING PROTEINS

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SUMMARY

We have employed high-performance chromatofocusing (HPCF) and high-performance size exclusion chromatography (HPSEC) to separate and identify radiolabelled estrogen binding proteins present in human uterine cytosol. Results obtained using these high-performance methods are compared to results of similar analyses by conventional isoelectric focusing procedures and open-column size exclusion chromatography. By HPCF, descending pH gradients (pH 8–4) allow us to discern four to five estrogen binding proteins with elution pH values typically between pH 4.5 and 7.2. However, when using HPCF, significant quantities of estrogen binding proteins are rarely detected between pH 7.2 and 8.0. This observation has been confirmed by open-column chromatofocusing of these proteins on Polybuffer Exchanger 94. In contrast, preparative isoelectric focusing by electrophoresis in polyampholytes reveals substantial quantities of estrogen binding activity eluted between pH 7.5 and 8.0. Several possible explanations for this disparity are discussed. Apparent differences are also observed when the size heterogeneity of estrogen binding proteins is analyzed simultaneously by size exclusion chromatography in open-column (Sephacryl S-300) and high-performance (TSK-3000SW) modes. HPSEC of these estrogen binding proteins on TSK-3000SW columns demonstrates a predominant 80–85 Å species, whereas size exclusion chromatography on conventional Sephacryl S-300 columns reveals two to three distinct regions of estrogen binding proteins with Stokes radii of *ca.* 85, 60 and 30 Å (major species). The larger form of receptor, whether a non-specific aggregate or a multisubunit complex, is stable in unfractionated cytosol and becomes more labile only during size exclusion chromatography.

INTRODUCTION

Steroid receptor proteins are unstable macromolecules present in endocrine target cells in minute quantities. They are routinely measured only by their ability to interact reversibly in a specific fashion with radiolabelled steroids. Chromatographic and physicochemical analyses of steroid receptor preparations under a variety of conditions have revealed an extensive and variable size and surface charge hetero-

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geneity[1–13]. Collectively, these properties have made steroid receptor proteins very difficult to purify and the basic mechanism of steroid hormone action is still not well understood.

The development of high-performance chromatofocusing (HPCF) has enhanced our ability to characterize rapidly the native surface charge properties of such labile macromolecules as steroid–receptor complexes[1]. However, as we will show here, our results with HPCF differ from those obtained by open-column chromatofocusing and isoelectric focusing by electrophoresis in ampholytes. Similarly, we have discovered rather significant differences in the apparent size/shape distribution of estrogen binding proteins when comparing open-column size exclusion chromatography (Sephacryl S-300) with high-performance size exclusion chromatography (HPSEC) on TSK-3000SW columns.

The results of our attempts to account for the diverse chromatographic behavior of estrogen receptor proteins and our specific recommendations for analyses of steroid–receptor complexes by high-performance liquid chromatography (HPLC) should be generally applicable to those characterizing the chromatographic behavior of other reversible protein–ligand complexes.

EXPERIMENTAL

Materials

Human uteri were obtained from patients undergoing hysterectomy for benign disease. The uteri were used immediately after rinsing in ice-cold saline. SynChropak AX-500 high-performance anion-exchange columns (250 × 4.6 mm I.D.) used for chromatofocusing were kindly provided by SynChrom. Altex TSK-3000SW high-performance size exclusion columns (600 × 7.6 mm I.D.) were purchased from Beckman. [^{3}H]Estradiol-17β (90–100 Ci/mmol) and [16α-^{125}I]iodoestradiol-17β ($\approx$1500 Ci/mmol) were purchased from New England Nuclear and Radiochemical Centre, Amersham, respectively. Trizma base, diethylstilbestrol (DES), dithiothreitol (DTT) and glycerol were from Sigma. Disodium ethylenediaminetetraacetic acid (2Na–EDTA) was from Fisher Scientific. Sephacryl S-300, Sephadex G-25, Sephadex G-25 PD-10 columns (prepacked), Polybuffer Exchanger 94, Polybuffer 96 and Polybuffer 74 were obtained from Pharmacia.

Preparation of cytosol and labelling of estrogen binding proteins

All procedures were performed in a cold-room at 0–6°C. Fresh uteri were rinsed in ice-cold saline and homogenized in two to four volumes of 25 m*M* Tris–HCl buffer (pH 7.4–7.8 at 0°C) containing 1 m*M* DTT and 20% (v/v) glycerol. Cytosol was obtained by high speed centrifugation (100,000 *g*; 60 min) of the homogenate and labelled at 0°C with 2–20 n*M* [^{3}H]estradiol-17β or [^{125}I]iodoestradiol-17β in the presence (non-specific binding) or absence (total binding) of a 100-fold molar excess of diethylstilbestrol (DES). Immediately before chromatofocusing or size exclusion chromatography, radiolabelled steroid–protein complexes were quickly (< 5 min) separated from excess of free steroid by rapid chromatography on small columns (9 ml) of Sephadex G-25 (Pharmacia PD-10 columns).

Chromatofocusing

The principles of chromatofocusing have been outlined previously[14–18]. The

relatively recent availability of macroporous high-performance anion-exchange columns (*e.g.*, refs. 19, 20), has allowed transfer of chromatofocusing principles to the high-performance mode. The development of HPCF for analysis of steroid–receptor complexes as well as details of this procedure were described earlier by Hutchens *et al.*[1]. High-performance AX-500 anion-exchange columns (250 × 4.6 mm I.D.) and open columns (9 × 0.7 cm I.D.) of Pharmacia Polybuffer Exchanger 94 (PBE 94) were equilibrated with 25 m*M* Tris–HCl buffer, containing 1 m*M* DTT and 20% glycerol adjusted to pH 8.0 at 0°C. All chromatography was carried out in a cold-room at 0–6°C. Samples of 250–1000 μl were loaded onto the columns and 1.0-ml fractions were collected at flow-rates of 0.5 ml/min (PBE 94) or 1.0–1.5 ml/min (HPCF). Elution was with linear pH gradients developed using Pharmacia Polybuffer 96 and 74 (30:70) diluted 1:15 in 20% glycerol (pH 3–4 at 0°C). The pH values of alternate fractions were determined immediately after chromatography at 0°C using a Corning Model 125 pH meter equipped with a micro combination calomel electrode. Radioactivity was determined either by scintillation counting (beta) using a Beckman Model LS 250 scintillation counter or directly (gamma) using a Packard Model 5220 Auto-Gamma Scintillation Spectrometer.

Size exclusion chromatography

High-performance size exclusion chromatography (HPSEC) was performed using an Altex TSK-3000SW column (600 × 7.6 mm I.D.) and a Perkin-Elmer Series 10 HPLC pump in a cold room at 0–6°C. Cytosolic estrogen binding proteins labelled with [^{3}H]estradiol-17β were cleared of excess of free [^{3}H]estradiol-17β by rapid chromatography on small (9-ml) Sephadex G-25 columns (PD-10). Aliquots (200 μl) were injected with Rheodyne 7125-S injectors and 1.0-min fractions were collected at 0.35 or 0.40 ml/min. Conventional open-column size exclusion chromatography was carried out using Sephacryl S-300 columns (95 × 1.6 cm I.D.) in a cold-room at 0–6°C. Samples (0.5–2.0 ml) were applied and 970-μl fractions were collected at 10 cm/h. The size exclusion columns (both S-300 and TSK-3000SW) were equilibrated with 50 m*M* potassium phosphate buffer (pH 7.4 at 0°C) containing 1.5 m*M* 2Na–EDTA, 1 m*M* DTT and 10% (v/v) glycerol and calibrated with six to nine purified proteins of known Stokes radii ranging from 18 to 86 Å. Column void volumes, V_0, were determined by exclusion of Blue-Dextran 2000. The column calibration was checked periodically and plots of log Stokes radii *versus* retention time (or elution volume) were linear with correlation coefficients (r values) typically better than 0.95.

RESULTS

Chromatofocusing of estrogen binding proteins: a comparison of HPCF using AX-500 columns with open-column chromatofocusing on PBE 94

This comparison was made to help determine the potential influence of support material composition on the separation of estrogen binding proteins during chromatofocusing. Variables to be considered include (1) silica-based (AX-500) *versus* agarose-based (PBE 94 is cross-linked Sepharose 6B)[18] charge support materials, (2) different effective pore diameters and (3) charge capacity.

Figs. 1 and 2 are representative profiles of human uterine estrogen binding proteins separated by HPCF using high-performance AX-500 anion-exchange col-

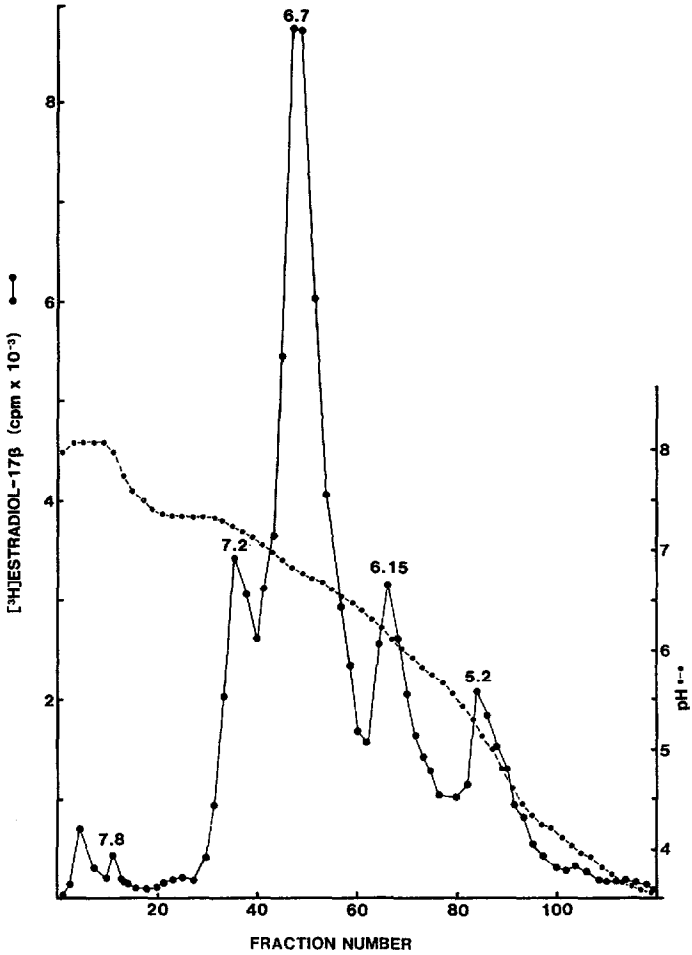

Fig. 1. Separation of [^{3}H]estradiol-labelled estrogen binding proteins from human uterus by high-performance chromatofocusing (HPCF) on AX-500. Cytosol was prepared from a fresh specimen of premenopausal human uterus and labelled with 20 n*M* [^{3}H]estradiol-17β. Termination of the labelling reaction (16 h at 0°C) and details of HPCF are described under Experimental.

umns. In Fig. 1, estrogen binding proteins in premenopausal uterine cytosol were labelled with 20 n*M* [^{3}H]estradiol-17β for 16 h at 0°C before HPCF analyses. The ability to generate this type of profile appears independent of the choice of ligand and menopausal status of the uterine tissue. As shown in Fig. 2, similar results were obtained when 2 n*M* [^{125}I]iodoestradiol-17β was similarly used to label estrogen binding proteins in cytosol prepared from postmenopausal human uteri. Fig. 2 also shows inhibition of specific estrogen binding sites when the same cytosol was incubated with the [^{125}I]iodoestradiol-17β in the presence of 2 μ*M* DES —an unlabelled competitor with high affinity and specificity for estrogen receptor proteins. The excellent reproducibility of HPCF is partly demonstrated in Fig. 2 by the near overlapping development of pH gradients in the sequential analyses shown. There is also

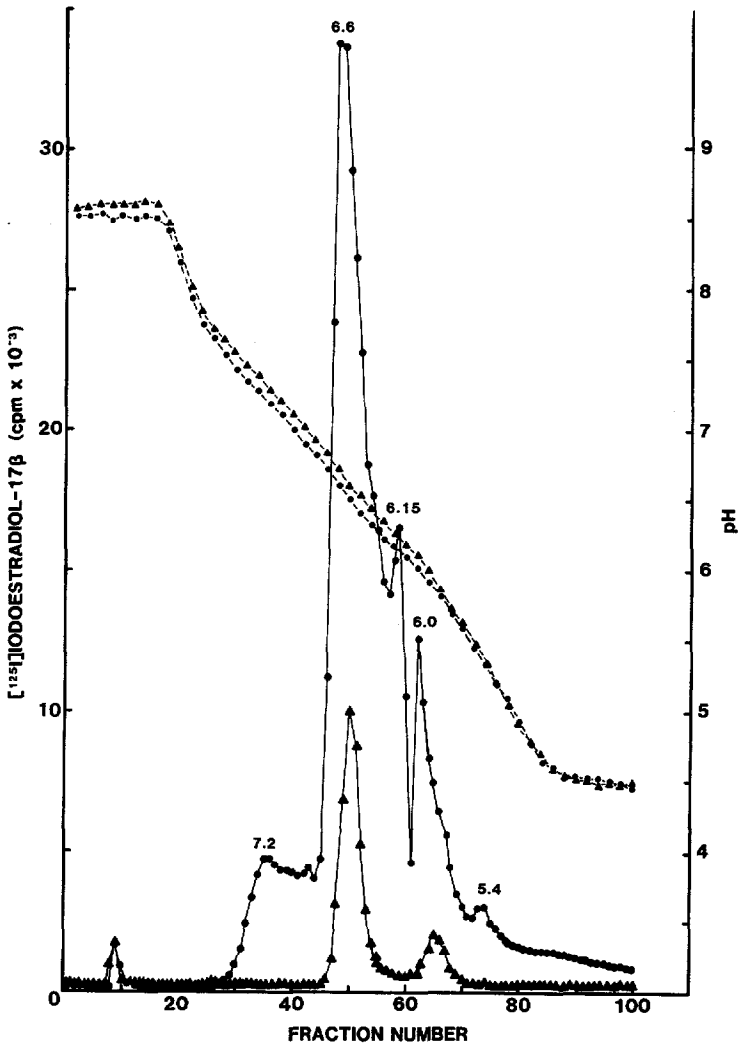

Fig. 2. Separation of [^{125}I]iodoestradiol-labelled estrogen binding proteins from human uterus by HPCF on AX-500. Cytosol was prepared from a frozen specimen of postmenopausal human uterus and labelled with 2 n*M* [^{125}I]iodoestradiol-17β both in the absence (●; total binding) and presence (▲; non-specific binding) of an unlabelled competitor, DES. Termination of the labelling reaction (24 h at 0°C) and details of HPCF are described under Experimental.

a striking similarity between the results shown in Figs. 1 and 2 and those obtained earlier during the development of HPCF for examination of molybdate effects on receptor surface charge[1]. Since different AX-500 columns, HPLC systems and tissues were used in the previous study, this helps to confirm both the technique and column consistency. The HPCF profiles of Figs. 1 and 2 reveal a lack of appreciable activity eluted before the peak at pH 7.1–7.2. This is in contrast to results obtained when similar preparations are analyzed by open-column chromatofocusing on minicolumns of PBE 94. Fig. 3 presents the profile obtained when postmenopausal uterine

cytosol was labelled with 10 n*M* [^{3}H]estradiol-17β and chromatofocused on open columns of PBE 94. The large peak of radioactivity eluted in the flow-through region before initiation of the pH gradient is typical of PBE 94 chromatofocusing results when cytosolic estrogen binding proteins are analyzed and represents free steroid and/or non-specific binding components (see below). In separate experiments, estrogen binding proteins in cytosol prepared from lactating rat mammary glands were analyzed simultaneously by HPCF on AX-500 columns and by chromatofocusing on minicolumns of PBE 94 and similar results were obtained[21]. There is a consistent presence of non-specific estrogen binding activity in the flow-through fractions when estrogen binding proteins are chromatofocused on PBE 94, which is not detected when the same preparations are similarly analyzed by HPCF using AX-500 columns.

Interaction of steroid with column material during chromatofocusing: a comparison of AX-500 and PBE 94

We have discovered that the affinity of steroid for the silica-based AX-500 HPCF column material may partially explain portions of the experimental results presented in the preceding section. As shown in Fig. 4, free steroid alone does not

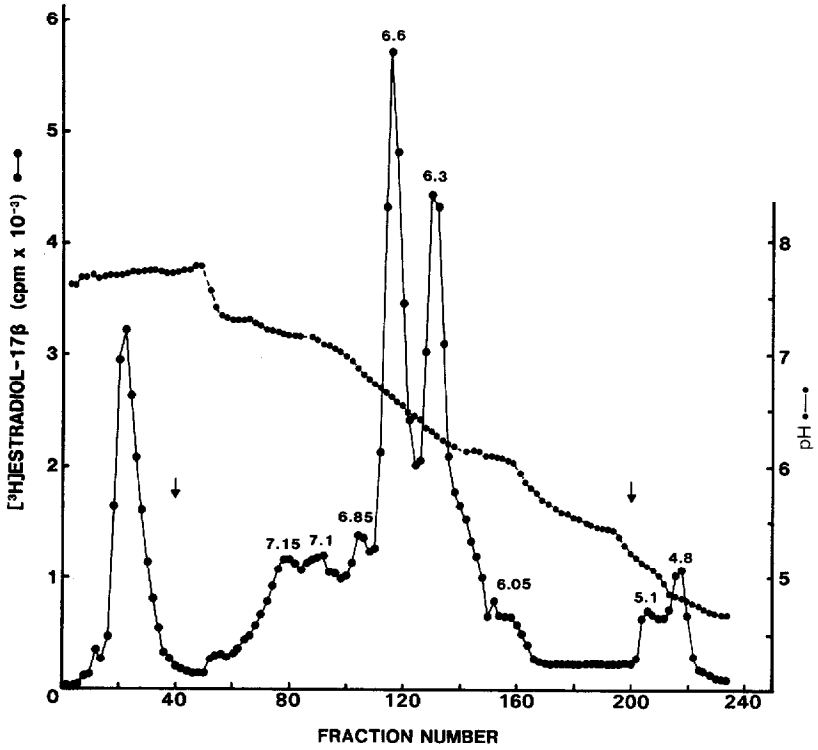

Fig. 3. Open-column chromatofocusing of human uterine estrogen binding proteins on PBE 94. Cytosol was prepared from a fresh specimen of postmenopausal human uterus and labelled with 10 n*M* [^{3}H]estradiol-17β. Termination of the labelling reaction and details of chromatofocusing on PBE 94 are described under Experimental. Arrows mark initiation of the primary pH gradient [Polybuffers 96 and 74 (30:70) diluted 1:15, pH 4 at 0°C] and secondary eluent (Polybuffer 74 diluted 1:15, pH 3 at 0°C), respectively.

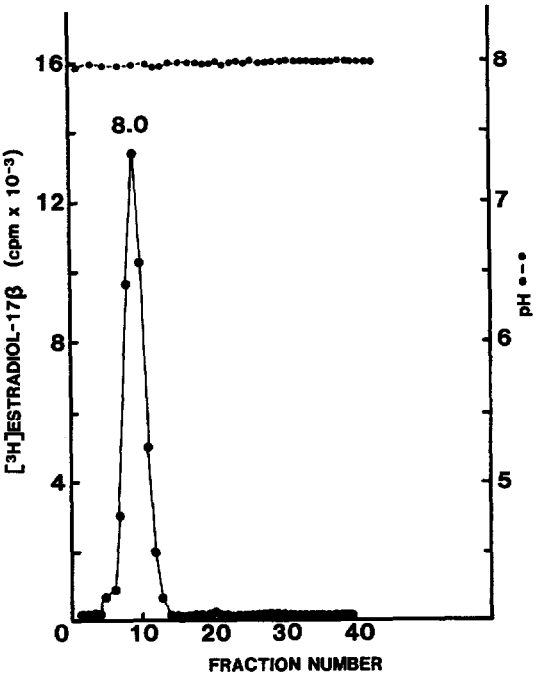

Fig. 4. Elution of free [^{3}H]estradiol-17β during open-column chromatofocusing on PBE 94. Unbound [^{3}H]estradiol-17β in homogenizing buffer was applied to a PBE 94 column and eluted as described under Experimental.

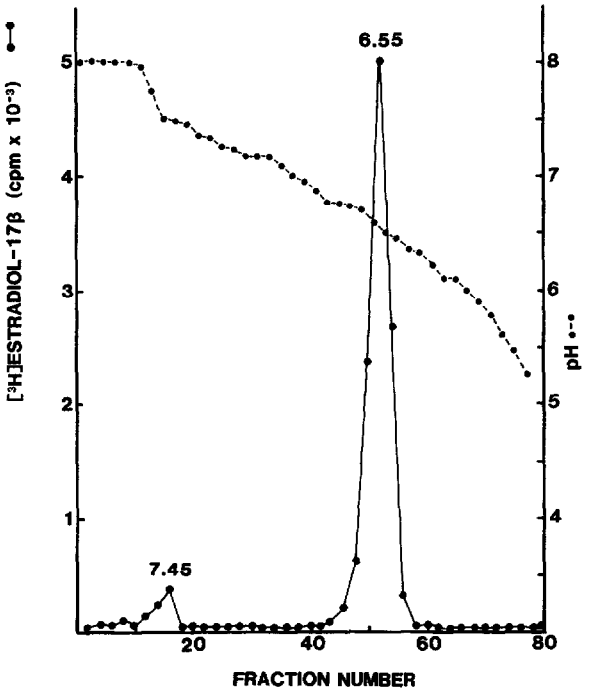

Fig. 5. Elution of free [^{3}H]estradiol-17β during HPCF on AX-500. Unbound [^{3}H]estradiol-17β in homogenizing buffer was applied to the AX-500 column and was eluted in a pH-dependent manner as described under Experimental.

react with PBE 94 and is eluted quantitatively in the flow-through region well before initiation of the pH gradient. Similarly, low-affinity, non-specific estrogen binding components in cytosol, labelled in the presence of excess of DES, are also eluted in the flow-through region when chromatofocused on PBE 94 (data not shown). This is in contrast to results shown for non-specific estrogen binding proteins, analyzed by HPCF on AX-500 (see Fig. 2). However, when cytosol is analyzed by HPCF in the absence of radiolabelled steroid and the individual fractions are subsequently incubated with [^{3}H]estradiol-17β (postlabelling), preliminary results suggest that

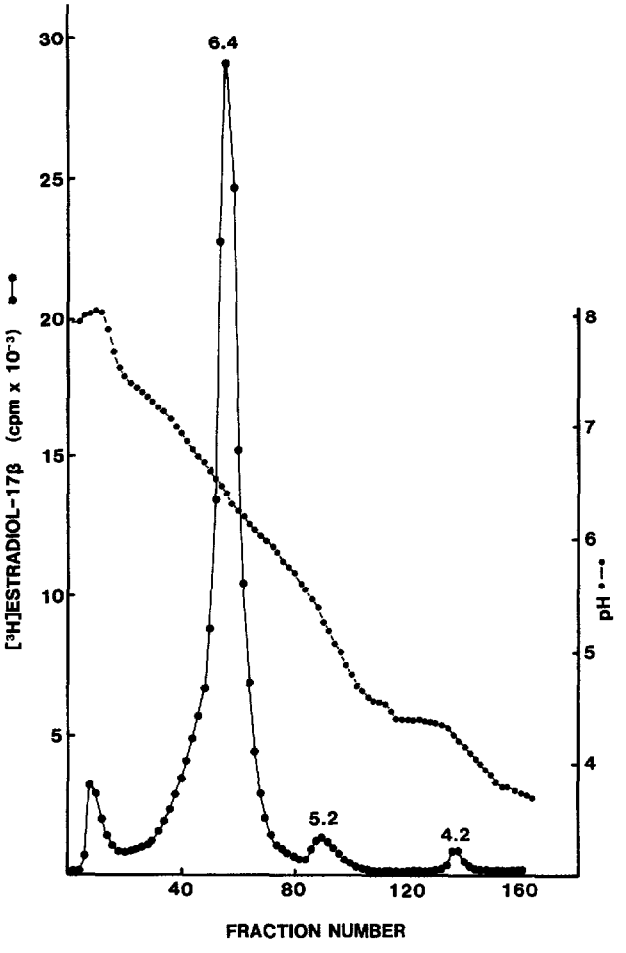

Fig. 6. Elution of [^{3}H]estradiol-17β during HPCF of serum estrogen binding proteins on AX-500. Serum from a premenopausal woman at mid-follicular phase (serum estradiol level = 386 pg/ml) was diluted in two volumes of homogenizing buffer and labelled for 16 h at 0°C with 20 n*M* [^{3}H]estradiol-17β in the presence (shown) and absence (not shown) of a 100-fold molar excess of unlabelled competitor, DES. Immediately before HPCF, unbound steroid was removed by rapid chromatography on small (9-ml) Sephadex G-25 columns. A 200-μl aliquot of the serum containing protein-bound [^{3}H]estradiol-17β was analyzed by HPCF on AX-500 columns as described under Experimental. The profile shown was for serum labelled with [^{3}H]estradiol-17β in the presence of excess of DES. No additional peaks of activity were detected when serum was labelled with [^{3}H]estradiol-17β alone thus indicating the "non-specific" nature of this binding.

non-specific, *i.e.* low-affinity, binding proteins are indeed present in the AX-500 flow-through fractions (data not shown). The interpretation of these results is further clarified by analysis of the free [^{3}H]estradiol-17β interaction with AX-500 columns. Fig. 5 shows that in contrast to PBE 94, free steroid interacts strongly with the AX-500 column material and is eluted in a pH-dependent manner at pH 6.5–6.6. The interaction and pH-dependent release of both [^{3}H]estradiol-17β and [^{125}I]iodoestradiol-17β from AX-300 anion-exchange columns was reported earlier[1]. A comparison shows how the elution pH of free steroid varies with individual HPCF columns. Fig. 6 shows the elution of [^{3}H]estradiol-17β from AX-500 during HPCF of low-affinity, serum estrogen binding proteins. Diluted serum was labelled with [^{3}H]estradiol-17β (in the presence and absence of excess of DES) and cleared of free steroid by G-25 chromatography immediately before analysis by HPCF on AX-500. The small peaks at pH 5.2 and 4.2 most likely represent testosterone–estrogen binding glubulin (TEBG) and serum Estrogen Binding Protein, respectively[2]. The activity eluted at pH 6.4 probably represents free steroid which is dissociated from the low-affinity serum estrogen transport proteins.

TABLE I

HUMAN UTERINE ESTROGEN BINDING PROTEINS: A COMPARISON OF FOCUSING RESULTS

Ref.	*Isoelectric focusing by electrophoresis (elution pH)*	*Open-column chromatofocusing (elution pH)*	*High-performance chromatofocusing (elution pH)*
2	4.0–4.4*	4.8	< 5.1**
	5.0–5.2***	5.1–5.4	5.1–5.3
	5.8–6.2	6.3	6.15
	–	6.6	6.6
	–	6.9	–
	–	7.1	7.1–7.2
	–	7.15	–
	7.5–8.0	–	–
3	4.8**		
	5.4		
	6.3		
	6.4		
	6.8		
	6.9		
4	4–5		
	6–7		
6	5.6–6.2		
10	6.3–6.4		
12	< 5		
	5.6		
	5.8		
	6.5		
22	6.15		

* Elution pH of estrogen binding proteins[2].
** Only in the presence of molybdate[1] and diisopropylfluorophosphate[3].
*** Elution pH of sex hormone binding globulin[2,22].

Estrogen receptor surface charge heterogeneity: a comparison of focusing results

Chromatofocusing (in both open-column and HPCF modes) provides an analytical as well as preparative means of rapidly separating estrogen receptor species of varying surface charge both in the presence and absence of receptor-stabilizing agents[1]. However, we have observed what may be an important difference between results obtained by chromatofocusing and those obtained when conventional isoelectric focusing by electrophoresis in polyampholytes is used[2–4,6,10,12,22]. A summary of focusing results is presented in Table I. Aside from the flow-through fractions, chromatofocusing by either mode yields results which are not too dissimilar. In both cases (as well as for isoelectric focusing) a major portion of specific estrogen binding activity appears at pH 6.3–6.7. Lesser amounts of activity are present at pH 7.1–7.2, 6.0–6.2 and near 5. However, by chromatofocusing, no specific estrogen binding activity (receptor) is present in fractions between pH 7.5 and 8.0. This is a consistent

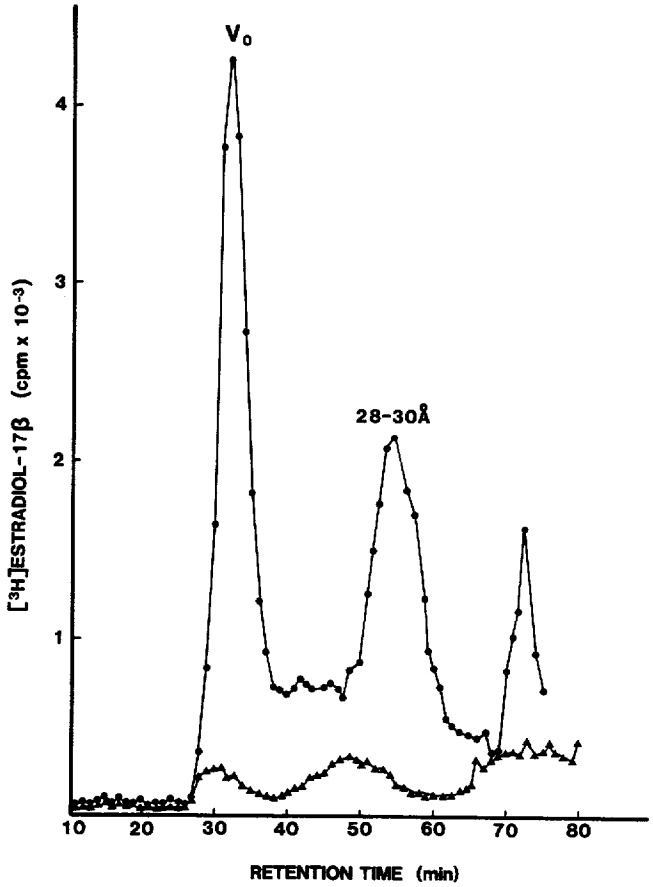

Fig. 7. High-performance size exclusion chromatography (HPSEC) of human uterine estrogen binding proteins on TSK-3000SW. Cytosol was prepared from a freshly obtained premenopausal uterus and labelled with 20 n*M* [^{3}H]estradiol-17β both in the absence (●; total binding) and presence (▲; non-specific binding) of an unlabelled competitor, DES (2 μM). Termination of the labelling reaction (6 h at 0°C) and details of HPSEC are described under Experimental. The void volume, V_0, and apparent Stokes radii of radiolabelled samples are indicated.

observation and stands in contrast to important results obtained by isoelectric focusing during electrophoresis in polyampholytes[2].

Size exclusion chromatography: a comparison of HPSEC on TSK-3000SW columns with open-column size exclusion chromatography on Sephacryl S-300

Size exclusion chromatography in the open-column (Sephacryl S-300) and high-performance (TSK-3000SW) modes yield different results regarding the size and/or shape heterogeneity of human uterine estrogen binding proteins. Fig. 7 is representative of results obtained when human uterine estrogen binding proteins are analyzed by high-performance size exclusion chromatography (HPSEC) on the TSK-3000SW columns. Two regions of estrogen specific binding activity are observed, namely, at the void volume, V_0, and at 55–56 min. By interpolation, using the column calibration profile, the elution volume of the latter species (55–56 min) suggests an apparent Stokes radius of 28–30 Å. This peak normally represents less than 40–50% of the total activity, the remainder being eluted at V_0. Fig. 8 demonstrates that the apparent size distribution of estrogen binding proteins is a function of how this parameter is determined. Sephacryl S-300 chromatography of human uterine estrogen binding proteins reveals an altogether different profile, some activity

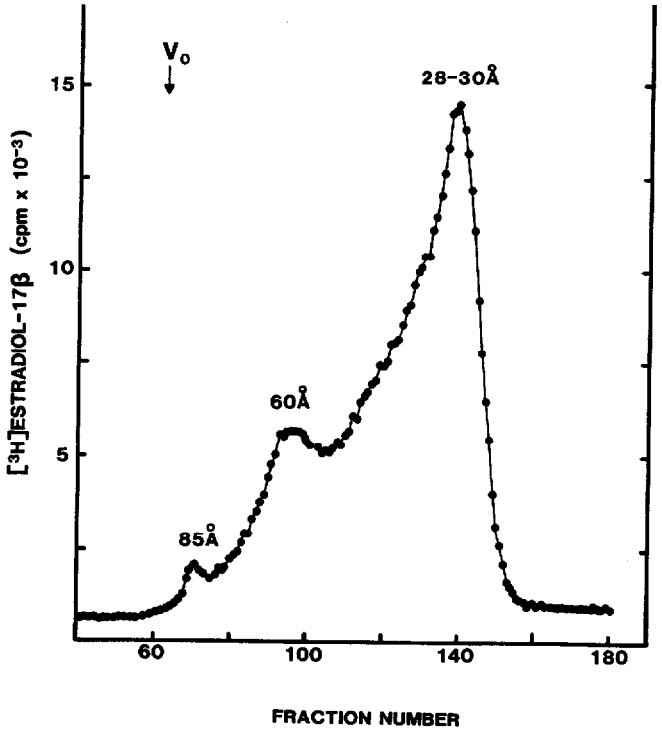

Fig. 8. Open-column size exclusion chromatography of human uterine estrogen binding proteins on Sephacryl S-300. Cytosol was prepared from a premenopausal uterine specimen and labelled with 20 n*M* [^{3}H]estradiol-17β for 6 h at 0°C. Termination of the labelling reaction and details of chromatography were as described under Experimental. The void volume, V_0, and apparent Stokes radii of radiolabelled samples are indicated.

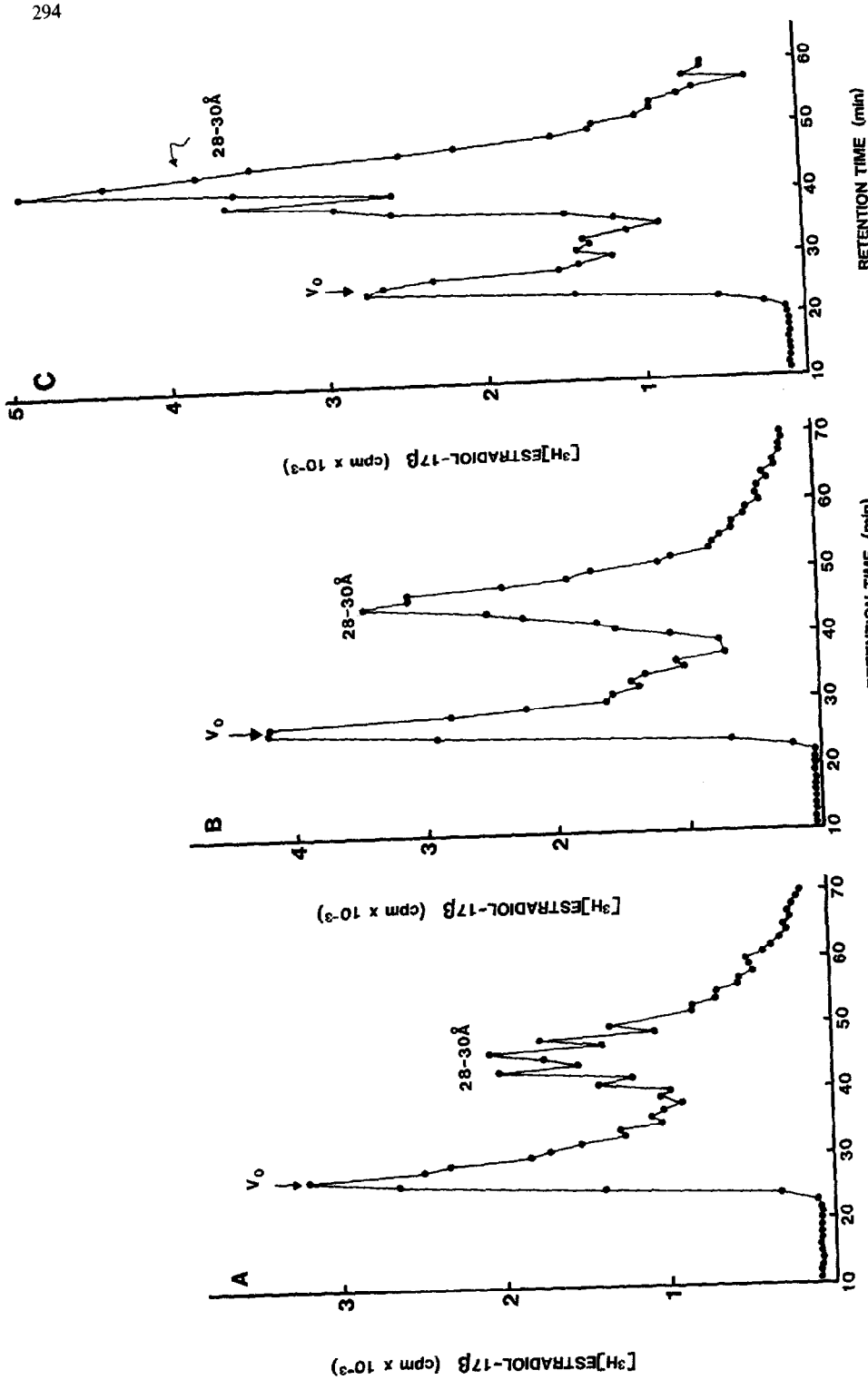

Fig. 9. Sequential analyses of human uterine estrogen binding proteins by HPSEC on TSK-3000SW. Cytosol was prepared from a fresh specimen of postmenopausal uterus and labelled at 0°C with 10 n*M* [^{3}H]estradiol-17β for various periods of time. Aliquots of cytosol were cleared of free steroid and analyzed after 28 (A), 46 (B) and 92 h (C) of incubation. Details of chromatography are described under Experimental. Void volumes and apparent Stokes radii of radiolabelled samples are indicated. The labelled cytosol analyzed by HPSEC as shown in A and B was also analyzed in parallel by open-column size exclusion chromatography on Sephacryl S-300 (see Fig. 10).

being eluted at 85 and 60–70 Å and the majority of activity being eluted at 28–30 Å. The skewed shapes of the peaks resulting from the relatively slower mode of size exclusion chromatography on Sephacryl S-300 suggests that intermolecular conversions (larger size to smaller size) are taking place during chromatography. The following experiments were designed to determine whether the relative ratio of these peaks were partly a function of total incubation time, since estrogen receptor processing and/or degradation events are known to occur in unfractionated cytosol[3–6,9,11,12]. The series of HPSEC profiles shown in Fig. 9 were generated from a single cytosol during prolonged incubation and show that the relative ratio of the two major regions of activity varies only minimally when cytosol preparations are analyzed after 28, 46 and 92 h of incubation. Certainly, the slight increase in relative quantity of the 28–30 Å species does not approach that observed by S-300 chromatography. The "large" form of receptor eluted at V_0 is relatively stable as a function of the total incubation time of unfractionated cytosol. The corresponding profiles shown in Fig. 10 represent Sephacryl S-300 size exclusion chromatograms of these *same* cytosols at the 28-h and 46-h incubation times. Again no really significant alterations in the shape or distribution of peaks are observed, yet they remain in striking contrast to the HPSEC profiles shown in Figs. 7 and 9.

DISCUSSION

Our observation that both free steroid and non-specifically bound estrogen are detected primarily (>90%) in the flow-through fractions during chromatofocusing on PBE 94 suggests that the other regions of activity eluted in a pH-dependent manner are estrogen receptor forms. The parallel analyses of estrogen-labelled human uterine cytosols on PBE 94 and AX-500 columns have helped to confirm the estrogen receptor status of the pH 6.6 peak observed during HPCF (Figs. 1 and 2). This is important due to the elution of free steroid (from AX-500 but not PBE 94) at or near this same pH.

Gibbons *et al.*[2] have recently described the physical separation of two kinetically distinct estrogen binding proteins in human uterine cytosols. One component, thought to be the "classical" estrogen receptor, has a high affinity for estradiol ($K_d = 10^{-10}$ *M*) and is eluted at pH 5–6 after preparative isoelectric focusing[23]. The other component has a slightly lower affinity for estradiol ($K_d = 10^{-9}$ *M*) and is eluted at pH 7–8 after isoelectric focusing. Both of these components were shown to be present in cytosol prepared from premenopausal uteri, whereas only the higher-affinity component was found in cytosols prepared from postmenopausal uteri. Our work towards purification of the different known forms of cytosolic estrogen receptor proteins[24,25], particularly by affinity chromatography, makes it imperative that we physically distinguish estrogen binding proteins with varying affinities for physiological concentrations of estradiol. Because of its superior resolving capabilities, rapid analysis times (60–90 min) and excellent recoveries (>90%), HPCF would appear to be the separation technique of choice[1,25]. HPCF has indeed proven useful in the separation of affinity-column purified estrogen-receptor proteins of various sizes[24,25]. However, to date, we have not observed an estrogen binding protein eluted at pH 7.5–8.0 that is directly analogous to the lower affinity estrogen binding protein seen in isoelectric focusing[2].

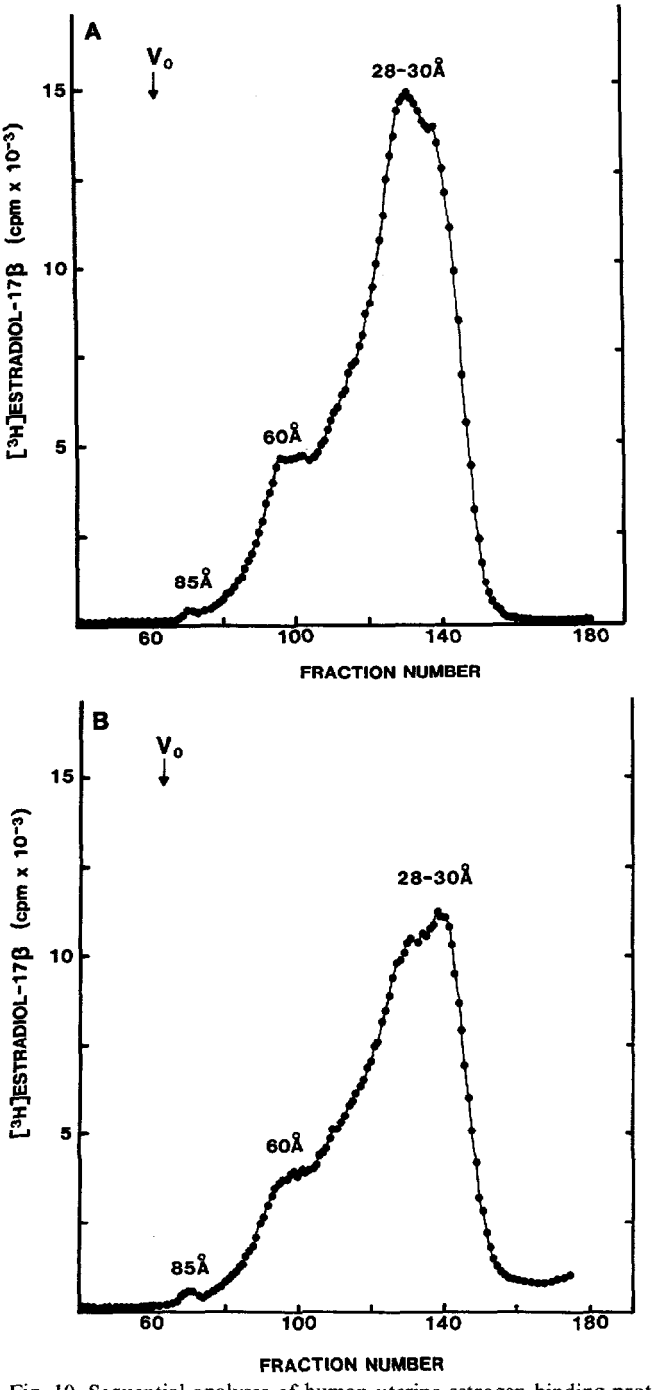

Fig. 10. Sequential analyses of human uterine estrogen binding proteins by open-column size exclusion chromatography using Sephacryl S-300. These profiles were obtained using the same preparation of cytosolic estrogen binding proteins analyzed in parallel by HPSEC to produce Fig. 9. A and B are representative of profiles obtained after 28 and 46 h of incubation, respectively. Details of chromatography are described under Experimental. The apparent Stokes radii of radiolabelled samples are indicated.

We have also observed what we feel to be technique-induced variations in the focusing behavior of estrogen receptor proteins from lactating rat mammary glands[21]. These variations might be due to purely innate methodological differences between chromatofocusing and isoelectric focusing by electrophoresis in polyampholytes. There are several theoretical considerations for potential differences in protein focusing results, *i.e.* 1–2 pH units, obtained by different means[14–18]. For example, under the non-denaturing conditions we must use, proteins of the same or nearly the same isoelectric point (p*I*) may undergo differential changes in surface charge as a function of pH and thus interact with a charged surface differently. Indeed, the interaction of a protein with a polyvalent column matrix may in itself alter the conformational response of that protein to changing pH. Furthermore the focusing effect brought about during chromatofocusing is the result of proteins repeatedly binding to and being released from the charged matrix. Therefore, both the concentration and composition of polyampholytes (competing anions in our case) may alter this interaction in a manner somewhat independent of pH. Clearly, this process will vary for different proteins. Finally, our observation of estrogen binding proteins eluted during HPCF at more acidic pH values than observed during isoelectric focusing may be accounted for by Donnan effects[14]. These effects may arise during chromatofocusing on highly charged (positive), porous matrices (both AX-500 and PBE 94) due to repulsion of positively charged species in solution, *i.e.*, ampholytes, hydrogen ions, metal cations, etc. Theoretically, the pH of the buffer immediately surrounding the surface of the porous matrix is likely to be lower than that at the surface as a result of cation repulsion. Therefore, regardless of the mechanism by which a protein is released from the matrix during chromatofocusing (ampholyte displacement or loss of net charge at p*I*), the protein will likely enter and finally be eluted at a pH relatively more acidic than that at which it was initially released.

Possible explanations for observed differences between the focusing of estrogen binding proteins by electrophoresis in polyampholytes and chromatofocusing may also be more artifactual than those discussed above. For instance, receptor isoforms or different species of estrogen binding proteins may be differentially labile during chromatofocusing *versus* isoelectric focusing[1,3,4]. This lability could be limited to the dissociation of steroid or be of a more irreversible, general nature. To address this possibility, we are currently using various preparations of estrogen receptor-specific monoclonal antibodies to detect estrogen receptor components independently of their ability to bind radiolabelled steroid[25].

Regarding HPSEC, the size/shape of cytosolic estrogen receptors prepared from calf, pig and rabbit uterus and lactating rat mammary glands has also been analyzed in parallel by Sephacryl S-300 chromatography and HPSEC using TSK-3000SW under identical conditions. Consistently, results obtained by these two techniques do not agree with respect to the quantity of specific activity in the void volume region[21,26,27]. Sucrose density gradient centrifugation of these same receptor proteins did not suggest the presence of a receptor form the size of that suspected at the void volume of a TSK-3000SW column[21,26,27]. The "large receptor" form, eluted in the void volume during TSK-3000SW chromatography, appears stable in cytosol but becomes labile during other, longer macromolecular separation processes, *e.g.*, sucrose density gradient centrifugation and S-300 chromatography. It may be artifactually aggregated or physicochemically excluded from the TSK-3000SW pore

matrices, *i.e.*, charge repulsion. Other investigators analyzing the size heterogeneity of steroid receptor preparations by HPSEC have observed "large forms" of receptor but have not reported parallel analyses by conventional size exclusion methodologies[9,28–30].

CONCLUSIONS

High-performance chromatofocusing (AX-500) of estrogen-binding proteins reveals profiles which differ from those obtained by open-column chromatofocusing (PBE 94), primarily because of the affinity of [^{3}H]estradiol-17β (free ligand) for the AX-500 silica-based support. Furthermore, and not surprisingly [^{3}H]estradiol-17β, associated with lower-affinity estrogen-binding proteins, *e.g.*, serum transport proteins, can become dissociated during HPCF analysis on AX-500. However, these proteins may be identified by subsequent incubation with [^{3}H]estradiol-17β (postlabelling). Parallel HPCF analyses of aporeceptor (ligand-free receptor) and holoreceptor (steroid–receptor complex) are possible and may be used not only to identify receptor proteins with lower affinity for steroid but also to identify steroid-dependent processing events, *i.e.*, subunit dissociation, which may take place during chromatofocusing of holoreceptor.

The rapid separation of estrogen-binding proteins by chromatofocusing suggests more extensive surface charge heterogeneity than is apparent from isoelectric focusing by electrophoresis in polyampholytes. Interestingly, analyses of uterine estrogen binding proteins by chromatofocusing (both open-column and high-performance modes) do not reveal significant quantities of the estrogen receptor species eluted at pH 7–8, discovered by isoelectric focusing[2]. This apparent disparity was also observed when estrogen receptor components in lactating rat mammary glands were analyzed in parallel by chromatofocusing and isoelectric focusing[21]. The nature of these differences is currently being investigated.

A comparison of size exclusion chromatography in open-column (S-300) and high-performance (TSK-3000SW) modes reveals significant variations in the distribution and relative ratio of estrogen binding proteins. These differences appear to arise during chromatography (protein separation) and are not the result of a simple time-dependent processing event in unfractionated cytosol.

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