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RAPID ANALYSIS OF ESTROGEN RECEPTOR HETEROGENEITY BY CHROMATOFOCUSING WITH HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Chromatofocusing principles have been utilized to develop a high-performance liquid chromatographic technique for the rapid and routine analysis of steroid receptor heterogeneity. Two anion-exchange columns (SynChropak AX-300 and AX-500) were compared for analytical and preparative chromatofocusing of estrogen receptor components. As many as ten different [^{125}I]iodoestradiol-labeled binding proteins were identified in cytosols prepared from mammary gland and uterus. Estrogen receptors were well separated from other cytosolic proteins and recovery of activity routinely exceeded 90%. Parallel analyses of these cytosols to determine receptor size and shape indicated that HPLC chromatofocusing can be used effectively to study receptor isoforms with Stokes radii ranging from 30 Å to greater than 70 Å. In contrast to isoelectric focusing, this technique is compatible with the inclusion of a commonly used receptor-stabilizing agent, sodium molybdate. Inclusion of molybdate during chromatofocusing of molybdate-stabilized receptor allowed the identification of two acidic receptor species not previously reported.

INTRODUCTION

Among the various chromatographic and electromigration techniques employed, isoelectric focusing has emerged as one of the most definitive techniques of protein separation. The availability of high-voltage, constant-power sources and efficient cooling systems has helped to reduce the long separation times and adverse temperature effects which once accompanied this procedure. Nevertheless, for small amounts of labile proteins, the usefulness of isoelectric focusing is severely limited by poor recoveries. Recently, a related technique, chromatofocusing, has been developed in which the focusing effect is produced by the generation of a chemically defined internal pH gradient on ion-exchange columns[1–4]. Although open-column chromatofocusing offers several distinct advantages over isoelectric focusing, separation times are routinely several hours[5]. The development of silica-based column materials having high mechanical stability, a porosity compatible with biological mac-

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romolecules.and a weak anion-exchange bonded phase has enabled the rapid separation of proteins in a manner analogous to classical DEAE-type ion-exchange chromatography[6]. We report here the development of very rapid chromatofocusing on these anion-exchange high-performance liquid chromatography (HPLC) columns in order to achieve high-resolution separations of small amounts (nanograms) Of cytosolic estrogen receptors.

Steroid hormone receptors are dynamic proteins which show a great degree of size, shape and charge heterogeneity[7–11]. The origin(s) of this molecular heterogeneity are foci of intense investigations. Certain structural as well as functional changes within the receptor macromolecule may actually be the result of manipulation *in* vitro, particularly during prolonged biochemical analyses'[2–14]. Therefore, a rapid separation technique which can be employed analytically and/or preparatively with high component recovery is needed to help understand the interrelationships of various receptor isoforms.

We have used HPLC chromatofocusing on AX-300 and AX-500 columns to study the heterogeneity of estrogen-receptor complexes in cytosols prepared from human uterus and lactating mammary glands from rat. Additionally, we have found that sodium molybdate, a widely used stabilizing agent for steroid receptors, may be included effectively in the column equilibration and elution buffers during HPLC chromatofocusing. This has provided the first opportunity of which we arc aware to focus molybdate-stabilized forms of steroid receptor macromolecules.

EXPERIMENTAL

Materials

The ligands (1 6α-[12 5]iodoestradiol-1 7β (1500–2000 Ci/mmol) and [^{3}H]estradiol-17β (90-I 15 Ci/mmol) were obtained from New England Nuclear. Disodium ethylenediamine-tetraacetic acid (EDTA), Tris–HCl and glycerol were obtained from Fisher Scientific. Unlabeled diethylstilbestrol (DES), Norit A, Dextran T-70, monothioglycerol, dithiothreitol and sodium molybdate were obtained from Sigma. Polybuffer 96 and Polybuffer 74 were purchased from Pharmacia.

The human uterine tissues used in this study were obtained from postmenopausal women through the aegis of surgeons and pathologists at local hospitals. Specimens were frozen and brought to the laboratory on dry ice or were placed on ice after surgery and brought to the laboratory. In either case, tissue was deep-frozen further in liquid nitrogen and stored at -86°C. Local pathologists confirmed the non-neoplastic nature of the tissue. Mammary glands were excised from Sprague-Dawley rats obtained from the Holtzman Company. These previously nulliparous animals were housed, bred, and delivered in the University vivarium. All animals delivered and nursed 9-14 pups and the day of delivery was designated the first day of lactation. At sacrifice, inguinal glands were removed and either used or frozen in liquid nitrogen. The frozen tissue was pulverized with a mortar and pestle at liquid nitrogen temperatures before storing it at -86°C.

Preparation of cytosolic estrogen receptors

All procedures were carried out at 0–4°C. Tissues or frozen powder were homogenized using a Brinkman Polytron (two 10-sec bursts) in 2–4 volumes of Tris ho-

mogenization buffer (10 m*M* Tris–HCl, 1.5 m*M* EDTA, 10% glycerol, 10 m*M* monothioglycerol, pH 7.4 at 4°C) with or without 10 m*M* sodium molybdate. Cytosols were prepared by centrifugation of the homogenates for 30-60 min at 40,000 rpm in a Beckman Ti 70.1 rotor. In the case of rat mammary gland, the supernatant lipid was separated from the cytosol layer. Since the receptor is present in cytosol in very low amounts, its detection depends upon the formation of a complex with its specific ligand, estradiol-17β. To this end, the cytosols were incubated at 4°C for 4-24 h with 1-3 n*M* [16α-^{125}I]iodoestradiol-17β in the presence (non-specific binding) or absence (total binding) of a 200-fold molar excess of an unlabeled competitor, DES. The incubations were terminated by removing unbound steroid in a pellet derived from an equal volume of a 1% dextran-coated charcoal suspension (1% charcoal, 0.5% dextran). The labeled cytosol was applied to the charcoal pellet, mixed briefly, allowed to stand for 5–10 min and then centrifuged at 600 g for 5–10 min to sediment the dextran-coated charcoal. Cytosol protein concentrations were determined by the methods of Waddell[15] or Bradford[16], using a kit from Bio-Rad Labs. Specific binding capacity was expressed as femtomoles of steroid bound per milligram of cytosol protein.

HPLC chromatojiicusing

All chromatography was performed in a cold-room at 0-6°C. Buffers were filtered under vacuum through Millipore 0.45-μm HAWP filters before use. Free steroid or the estrogen-labeled cytosols were applied to SynChropak AX-300 or AX-500 (250 × 4.1 mm I.D.) anion-exchange columns (SynChrom) with an Altex Model 210 sample injection valve (Beckman). Elution was carried out using Altex Model 112 pumps. The absorption profile of the eluate was monitored at 280 nm with a Hitachi 100-40 spectrophotometer equipped with an on-line flow cell (Beckman).

Two different column equilibration and elution programs were used depending upon the initial buffer conditions of the receptor preparations. The columns were initially equilibrated to the starting pH (slightly above the desired upper limit) using a common cationic buffer. In the case of HPLC chromatofocusing on AX-300 and AX-500 columns, we have used 25 m*M* Tris-HCl containing 1 m*M* dithiothreitol and 20% (v/v) glycerol adjusted to pH 8.1-8.3 at 0°C. For chromatofocusing molybdate-stabilized receptor components, 10 m*M* sodium molybdate was included in

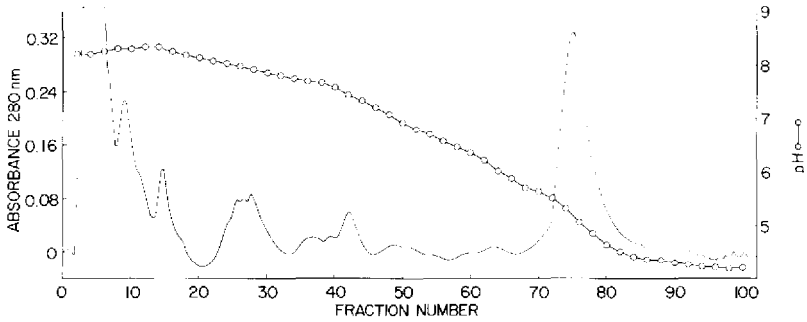

Fig. 1. HPLC chromatofocusing of cytosolic proteins from rat mammary gland after 14 days of lactation. Cytosol proteins (2–5 mg) were injected and eluted from the AX-300 column by a 30:70 mixture of Polybuffers 96 and 74, diluted 1:10 with 20% glycerol and adjusted to pH 4.5.

the column equilibration buffer. Cytosols prepared in Tris homogenization buffer were eluted with a 30:70 mixture of Polybuffers 96 and 74. This polyampholyte solution was diluted 10- to 20-fold with 20% glycerol, filtered with a 0.45-μm filter (Millipore) and adjusted to between pH 4.0 and 5.0 at 04°C. A representative pH gradient generated by this method is shown in Fig. 1. Various dilutions of Polybuffers 96 and 74 were utilized for each of the columns, as noted. The cytosols prepared in Tris homogenization buffer that additionally contained molybdate were eluted sequentially using two separate polyampholyte buffers. The primary eluent was a 30:70 mixture of Polybuffers 96 and 74 diluted 1:10 with 20% glycerol containing 10 m*M* sodium molybdate, filtered and adjusted to pH 5.0 at 0-4°C. The secondary eluent was Polybuffer 74 diluted 1:8 with 20% glycerol (no molybdate), filtered, and adjusted to pH 3.5 at 04°C. For all experiments shown, l.O-ml fractions were collected at 1.0 ml/min. Columns were regenerated to their starting pH (8.3) with column equilibration buffer at 1–2 ml/min (Table I). The [^{125}I]iodoestradiol-17β-labeled receptor complexes, non-specific binding components, and free steroid in each of the 1 -ml fractions were detected radiometrically in a Micromedics 4/600 gamma counter. The pH of alternate fractions was determined at 0°C using a Beckman Model 3500 pH meter with a combination glass electrode. The counting efficiency averaged 65%, as judged by reference to independent determinations of disintegrations per minute using a Beckman 4000 two-channel gamma counter [17].

RESULTS AND DISCUSSION

Characteristics of HPLC chromatofocusing

In chromatofocusing, proteins are focused during the generation of a chemically defined, internal pH gradient within the anion-exchange column. Our earlier chromatofocusing studies[18] in which Polybuffer Exchanger 94 (Pharmacia) was used, suggested that chromatofocusing may prove quite useful as an investigative tool to monitor alterations in receptor architecture and functional status resulting from varying experimental manipulations. However, elution times and column regeneration times were quite lengthy[5,18]. Therefore, we investigated the use of two different HPLC anion-exchange columns for rapid chromatofocusing. SynChropak AX-300 and AX-500 columns are silica-based, polyamine-coated gels which differ not only

TABLE I

OPTIMAL CONDITIONS FOR HPLC CHROMATOFOCUSJNG OF STEROID HORMONE RECEPTORS ON SYNCHROPAK AX-300 AND AX-500*

Parameter	*AX-300*	*AX-500*
pH gradient	8.3 4.5	8.3 4.5
Dilution of Polybuffers 96 and 74 (30:70)	1:10	1:20
pH decrease (units/ml)	0.06 ± 0.01	0.05 ± 0.01
Separation time (1 ml/min)	50 80 min	60–100 min
Regeneration time (2 ml/min)	60 120 min	20 30 min

* AX-300 and AX-500 are polyamine-coated silica gels (SynChrom) with pore diameters of 300 Å and 500 A, respectively.

in Pore size (Table I) but also in their effective ion-exchange capacities (93 and 59 mg bovine serum albumin/g gel, respectively, according to ref. 19).

The nature of the pH gradient formed during chromatofocusing is partly a function of the buffering capacity of the polyamine groups on the column, It is also a function of the buffering capacity of the eluent. The column is titrated to a pH which establishes the starting pH of the gradient. The Polybuffer eluent is adjusted to a pH which defines the lower limit of the desired gradient. We have been primarily interested in generating a relatively broad pH range in order to identify adequately heterogeneous receptor forms of widely varying elution pH values. Isoelectric focusing results with estrogen receptors from these tissues suggested that pH 8.3 to 4.5 might be appropriate[9,18].

In order to develop reproducible pH gradients within optimal separation times, several Polybuffer dilutions were investigated for both the AX-300 and the AX-500 columns. As shown in Table I, for pH gradients from approximately 8.3 to 4.5, we have found a 1:10 dilution of a 30:70 mixture of Polybuffers 96 and 74 to give good results with the AX-300 column (Fig. 1). This same dilution of Polybuffer (1:10) results in a steeper gradient (ΔpH/ml = 0.09 pH units/ml) when used with the AX-500 column. The steepness of the pH gradient generated for the AX-500 column can be decreased by using a more dilute Polybuffer solution. We have found that a 1:20 dilution of the Polybuffer 96-Polybuffer 74 (30:70) mixture results in reproducible pH gradients of dimensions similar to those obtained with the AX-300 column and 1:10 dilute Polybuffer (Table I). It is important to note that under these conditions, only half the amount of Polybuffer is needed per analysis. Furthermore -and this is important in work with labile proteins, such as receptors- the column regeneration is 2-6 times faster.

We are interested in the large-molecular-weight forms of steroid receptor proteins. These forms are currently thought to be either precursors[9,11,20] and/or specific complexes with RNA-containing macromolecules[11,21–24] or specific plasma membrane binding components[25,26]. We are therefore concerned with the effects of gel pore diameter on chromatofocusing results. It has been estimated that as much as 95% of the surface area of a porous support is inside the pore network[6,19]. If a macromolecule cannot effectively penetrate the pore matrix to reach this surface area, the severe reduction in mass transfer from the mobile to the stationary phase results in a loss of resolution. Even though the optimum relationship between pore diameter and sample size or shape is not available, preliminary studies by others[6] suggest that AX-300 columns (300-A pore diameter) should be used with macromolecules less than 10^5 daltons in size. Similar data for the AX-500 column are unavailable.

The receptor preparations used to generate the HPLC chromatofocusing profiles presented here have been analyzed simultaneously by sucrose density-gradient centrifugation and by size-exclusion chromatography on a Sephacryl S-300 column and/or HPLC on a TSK 4000 SW column (HPSEC). These parallel analyses confirm that the principal receptor species analyzed by HPLC chromatofocusing initially had sedimentation coefficients of 8-10 S and Stokes radii of at least 70 Å. These data allow the molecular weight of these receptor species to be estimated at over 300,000. Subsequent HPSEC analyses of the fractions eluted from chromatofocusing columns by the more acidic buffers suggest that even in the absence of molybdate the large size of the receptors is preserved. While this supporting evidence was not obtained

with Polybuffer, the ionic strength of the buffers used was equal to or greater than that of the diluted Polybuffer.

HPLC chromatofocusing of cytosol proteins

A representative profile of cytosol proteins, separated by HPLC chromatofocusing, is shown in Fig. 1. Cytosol was prepared from lactating mammary gland using Tris homogenization buffer, and 250 μl was applied to an AX-300 column, equilibrated to pH 8.3. The proteins (2 4 mg) were eluted at the rate of 1.0 ml/min by a 30:70 mixture of Polybuffers 94 and 74, diluted 1:10 with 20% glycerol and adjusted to pH 4.5. The gradient formed in this manner decreased by *ca.* 0.06 pH units/ml and allowed the detection of numerous protein components. The separation of cytosol proteins observed was better than that normally obtained with the DEAE-type anion-exchange procedures commonly used to evaluate receptor heterogeneity[9,13,27].

***HPLC** chromatofocusing of cytosolic estrogen-binding proteins from lactating mammary gland on* AX-300 *and AX-500*

Several distinct species of estrogen-binding proteins can usually be identified within a given cytosol. The profile shown in Fig. 2 represents data obtained when estrogen-labeled cytosol from lactating (14 days) mammary glands was chromatofocused by HPLC on AX-300. For this particular chromatofocusing analysis (Fig. 2), the Polybuffer eluent was adjusted to pH 5.0. The major peak of receptor activity observed at pH 5.4 was eluted very near the end of the pH gradient. Because of the relatively short time required to regenerate the AX-300 column with equilibration

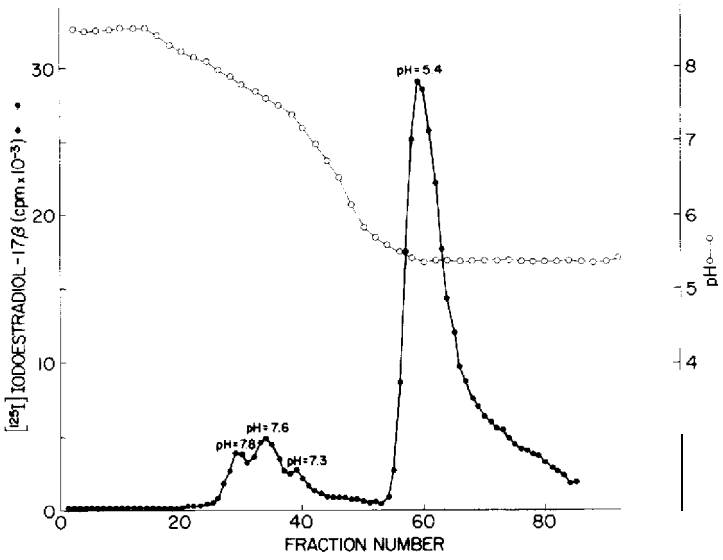

Fig. 2. Separation of receptor isoforms by HPLC chromatofocusing on AX-300. Cytosol was prepared from 14-day lactating mammary glands and incubated with [^{125}I]iodoestradiol-17β as described in the Experimental section. For elution a 30:70 mixture of Polybuffers 96 and 74, diluted 1:10 with 20% glycerol and adjusted to pH 5.0, was used.

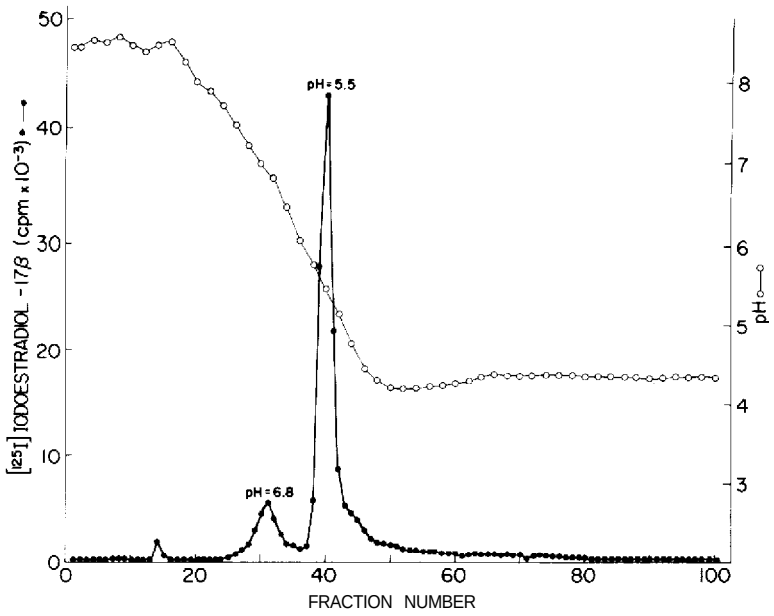

Fig. 3. Demonstration of variable receptor heterogeneity by HPLC chromatofocusing on AX-500. Cytosol was prepared from 14-day lactating mammary glands and incubated with [^{125}I]iodoestradiol-17β as described in the Experimental section. For elution a 30:70 mixture of Polybuffers 96 and 74, diluted 1:10 with 20% glycerol and adjusted to pH 4.0, was used.

buffer to its starting pH (Table I), the analysis could be repeated within 2-3 h. When this cytosol was subsequently again analyzed with a gradient which extended to pH 4.0, the peak of activity at 5.4 seen in Fig. 2 was sharply focused within the linear range of the gradient, thereby confirming its elution pH (data not shown).

The two main regions of receptor activity, namely pH 778 and pH 556, were also seen when this cytosol was analyzed by isoelectric focusing (data not shown). The relative proportion of activity in these two regions appears highly variable. We have generated AX-300 chromatofocusing profiles from similar receptor preparations where 40–60% of the observed activity was eluted between pH 7 and 8. Furthermore, each of the estrogen-binding components at pH 7-8 was shown to be associated with labeled steroid in a specific fashion when DES was used as an inhibitor. The variable presence and relative amounts of these receptor isoforms appear to be a function of mammary gland differentiation. These data are to be presented elsewhere.

The profile shown in Fig. 3 was generated by chromatofocusing on AX-500 and helps to illustrate the variable heterogeneity of cytosolic estrogen receptors. [^{125}I]Iodoestradiol-17β-labeled cytosol was prepared from rat mammary glands also at 14 days of lactation. The bulk of the activity was still sharply focused at pH 5.4–5.5. However, a significant peak of activity was now eluted at pH 6.8. In contrast to the profile shown in Fig. 2, little activity was eluted above pH 7.

The quantity of estrogen receptors present in the cytosols used to generate the chromatofocusing profiles shown in Figs. 2 and 3 was similar. Previous titration analyses[28] and parallel sucrose density-gradient analyses demonstrated that both cy-

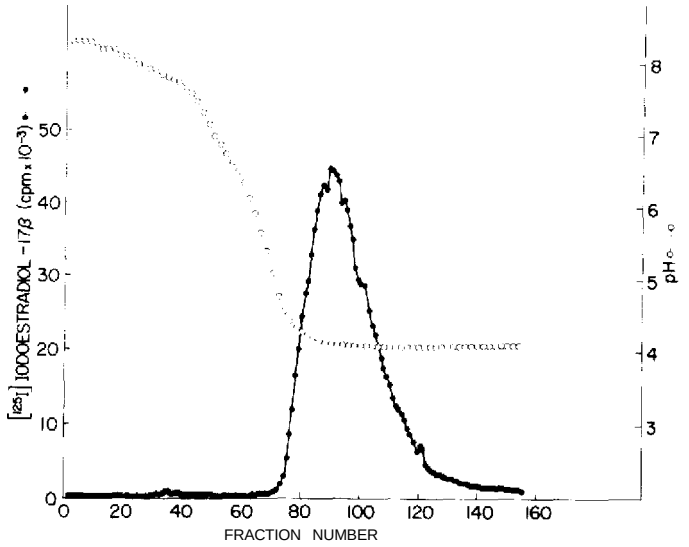

Fig. 4. Interaction of unbound [^{125}I]iodoestradiol-17β with AX-300. Labeled steroid was applied to the AX-300 column as a dilute solution in column equilibration buffer (see Experimental). For elution, see Fig. 3.

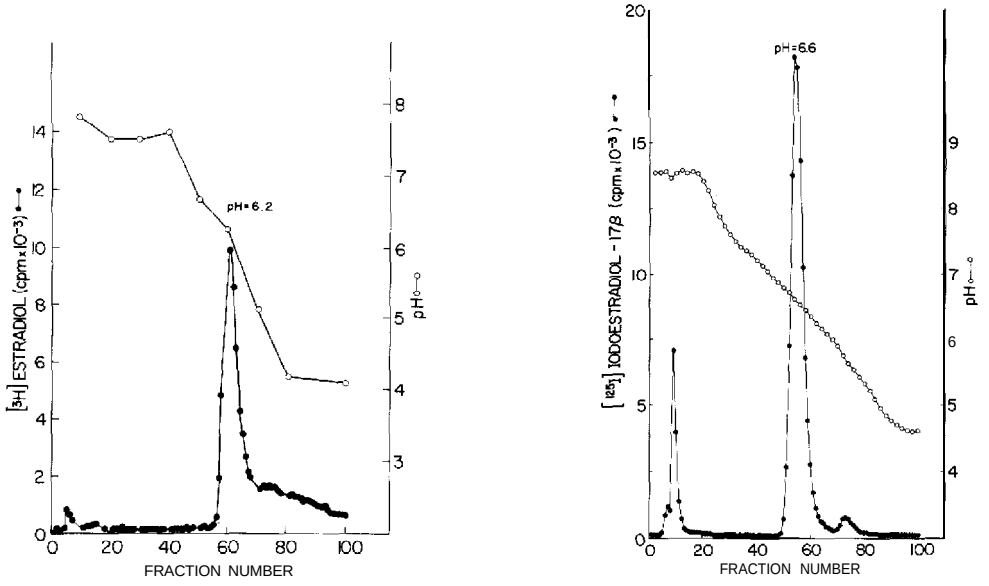

Fig. 5. Interaction of unbound [^{3}H]estradiol-17β with AX-300. Labeled steroid was applied to the AX-300 column as a dilute solution in column equilibration buffer (see Experimental). For elution, see Fig. 3. Alternate fractions (0.325 ml) were counted in a Beckman LS9000 liquid scintillation counter. Uncounted fractions adjacent to every tenth tube were pooled and the pH measured.

Fig. 6. Interaction of unbound [^{125}I]iodoestradiol-17β with AX-500. Steroid was applied to the AX-500 column as a dilute solution in column equilibration buffer (see Experimental). For elution a 30:70 mixture of Polybuffers 96 and 74, diluted 1:20 with 20% glycerol and adjusted to pH 4.5, was used.

tosols contained 20–30 fmole of receptor per milligram of cytosol protein. The presence of different receptor isoforms revealed here by rapid HPLC chromatofocusing may be of some clinical significance. Our laboratory suggested that the relative amounts of 4S and 8S receptor forms in breast cancers may more accurately predict patient response to hormonal or ablative therapies than the total amount alone[29].

Analysis of radioligand-receptor complexes required additional control experiments not necessary for studies of single components. Steroid receptor proteins are dynamic entities which exist *in vitro* in several different forms[7–14]. Unlike peptides and other proteins most commonly analyzed by HPLC, these various receptor isoforms have not been purified. Therefore, the only means of identifying these unique proteins is to use radiolabeled steroids of high specific activity. We routinely use both [^{125}I]iodoestradiol-17β and [^{3}H]estradiol-17β to label estrogen receptors in cytosols from various hormone target organs specifically. Although the affinity of these ligands for the estrogen receptor is high [K_d (apparent dissociation constant) = 10^{-10}–10^{-11} M], we have observed that manipulation of the steroid-receptor complex *in vitro (i.e.* purification) can result in a significant increase in the steroid dissociation rate (unpublished observation).

Since only radioactivity is monitored during analysis of receptor molecules, it is imperative to distinguish receptor-bound steroid from free steroid. As stated under Experimental, free steroid was removed from all cytosol receptor preparations before analysis by dextran-coated charcoal treatment. However, even though HPLC chromatofocusing is much more rapid than the majority of other analytical procedures, it was essential to confirm the elution behavior of unbound radioactivity fractionated during chromatofocusing, because steroid-receptor complexes may still dissociate during analysis. It can be seen in Fig. 4 that free [^{125}I]iodoestradiol-17β was bound to AX-300 and was eluted quantitatively in a pH-dependent manner at *ca.* pH 4.0. In separate chromatofocusing experiments with the AX-300 column where free [^{125}I]iodoestradiol-17β was used (not shown), neither extensive washing with equilibration buffer (pH 8.3) nor the application of a linear O-l.0 M salt gradient resulted in steroid elution. Similarly, tritium-labeled estradiol was bound by the AX-300 column, but eluted at cu. pH 6.2 (Fig. 5). In contrast to those results obtained with the AX-300 column (Fig. 4), Fig. 6 shows a different elution profile, observed when free [^{125}I]iodoestradiol-17β interacted with the AX-500 column. Presumably, the striking difference in the elution behavior of [^{125}I]iodoestradiol-17β between AX-300 and AX-500 is attributable to differences in the manufacture of the packing materials[30]. Therefore, when AX-500 and AX-300 are used in either chromatofocusing or conventional ion-exchange columns for analysis of steroid hormone complexes, considerable care seems advisable in the interpretation of radioactivity profiles.

Because of steroid interactions with these ion-exchange columns, we emphasize the importance of high component recoveries. Recoveries of the free ligand or ligand–receptor complexes from either the AX-300 or AX-500 columns typically exceed 90%. This eliminates some of the ambiguities associated with methods giving lower recoveries.

HPLC chromatofocusing of estrogen-binding proteins from human uterus on AX-500

The ion-exchange capacity of the AX-500 column is only *ca.* 60% of that of the AX-300 column[19]. Therefore, it may be predicted that within the same pH

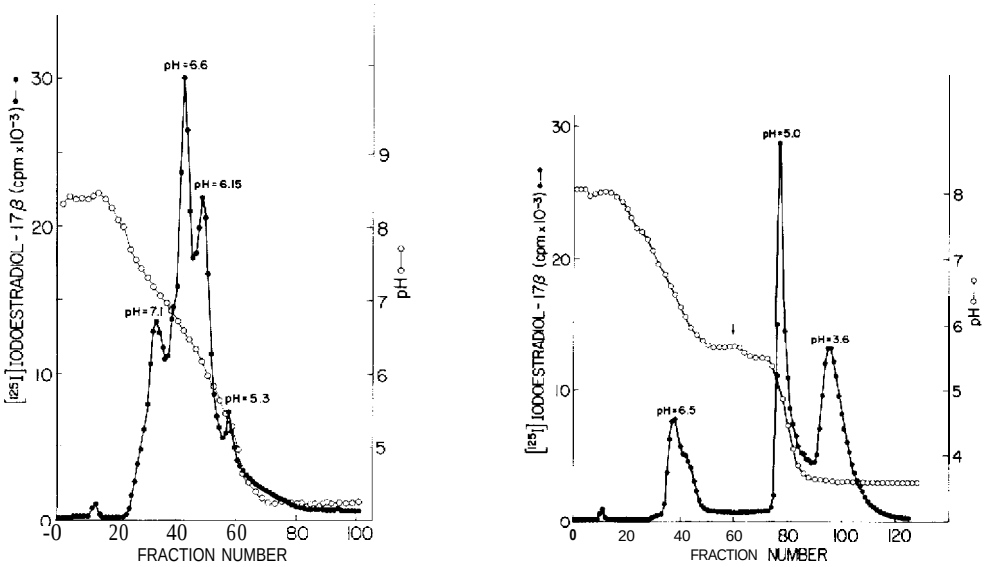

Fig. 7. Separation of uterine estrogen-receptor isoforms by HPLC chromatofocusing on AX-500. Cytosol was prepared from the uterus of a postmenopausal woman and incubated with [^{125}I]iodoestradiol-17β as described in the Experimental section. Activity was eluted with a 30:70 mixture of Polybuffers 96 and 74 diluted 1:15 with 20% glycerol and adjusted to pH 4.5. The recovery of activity in this representative experiment was 97%.

Fig. 8. Identification of molybdate-stabilized receptor species by HPLC chromatofocusing on AX-500 in the continued presence of molybdate. Human uterine cytosol was prepared and labeled with [^{125}I]iodoestradiol-17β in the presence of 10 mM sodium molybdate (see Experimental). Receptor preparations (2 4 mg) were eluted from the AX-500 column with a biphasic pH gradient. The primary eluent was a 30:70 mixture of Polybuffers 96 and 74, diluted 1:10 with 20% glycerol containing 10 mM sodium molybdate and adjusted to pH 5.0. The secondary eluent (initiated at arrow) was Polybuffer 74, diluted 1:10 with 20% glycerol (no molybdate) and adjusted to pH 3.5. The recovery of activity in this representative experiment was 91%.

range and for a given Polybuffer dilution, gradient development would occur more quickly with the AX-500 column than with the AX-300 column. We have found this to be the case (Table I). Thus, for AX-500 chromatofocusing we have been able to maintain good, reproducible results with Polybuffer elution mixtures diluted up to 1:15 or even 1:20.

Fig. 7 is a representative AX-500 chromatofocusing profile of estrogen-binding proteins in cytosol from human uterus. In this case, application of a 1:15 dilution of Polybuffer revealed their complex molecular heterogeneity. However, use of a 1:20 dilution resulted in a similar profile. Within 60 min, 95% of the activity was eluted. Typically, four distinct peaks of activity are apparent with pH values of 7.1-7.2, 6.6, 6.15 and 5.4–5.5 (n = 3). The peak at pH 6.6 is typically the predominant species. Portions of this same cytosol were incubated with [^{125}I]iodoestradiol-17β in the presence of excess radioinert competitor (DES) to determine the level of non-specific binding activity (see Experimental section). When this non-receptor estrogen-binding activity (only 5520% of total binding) was chromatofocused on an AX-500 column, it was eluted at pH 6.6 (data not shown). As shown in Fig. 6, free [^{125}I]iodoestradiol was also eluted primarily at pH 6.6 from AX-500 under these conditions.

It is possible that [^{125}I]iodoestradiol initially bound to the lower-affinity, non-receptor components in cytosol was dissociated during chromatofocusing and eluted as free steroid. Receptors in fractions of cytosol which have been chromatofocused in the absence of ligand may be detected by their subsequent association with [^{125}I]iodoestradiol-17β (postlabeling). This procedure is currently being examined to estimate more accurately the amount of estrogen receptor focused at the pH 6.6 position. This experimental approach may also reveal steroid-dependent changes in receptor actually brought about during chromatofocusing. This is a distinct possibility when anion-exchange columns are used[10,13]. Postlabeling of glucocorticoid receptors, fractionated by DEAE-cellulose chromatography in the absence of ligand has demonstrated that both structural and functional changes associated with receptor "activation" can result during chromatography of this receptor in the presence of its specific ligand[31].

HPLC chromatofocusing of molybdate-stabilized estrogen receptors on AX-500 in the continued presence of molybdate

The complex elution profile shown in Fig. 7 can be dramatically altered by employing a commonly used receptor stabilizing agent, sodium molybdate. Sodium molybdate has been previously shown to stabilize steroid association and to induce both size and charge stability of the receptor[9-11,13]. Since these receptor-stabilizing effects have been shown to be reversible[31–33], we needed a sensitive analytical method, compatible with the concentrations of molybdate normally used, usually 10 mM for monitoring them properly. In contrast to isoelectric focusing, this is possible with chromatofocusing.

To demonstrate this, other cytosols were prepared from the same human uterus used to generate the profile shown in Fig. 7. However, 10 mM sodium molybdate was included in the homogenization buffer. When analyzed by HPLC chromatofocusing on an AX-500 column also equilibrated with molybdate, profiles typified by

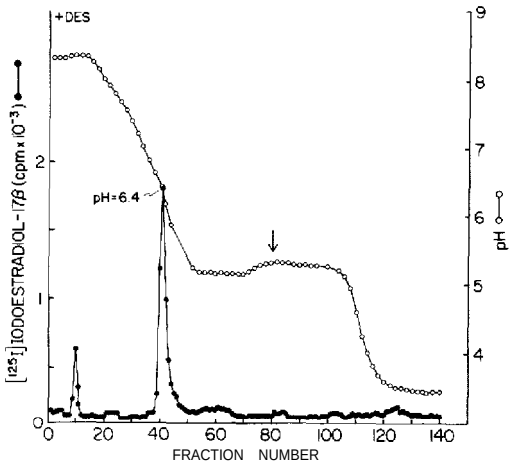

Fig. 9. Use of AX-500 to identify non-specific [^{125}I]iodoestradiol-17β binding remaining in molybdate-treated uterine cytosol after incubation with DES (non-specific binding as described in Experimental). The cytosol preparation and chromatography conditions were identical with those given for Fig. 8.

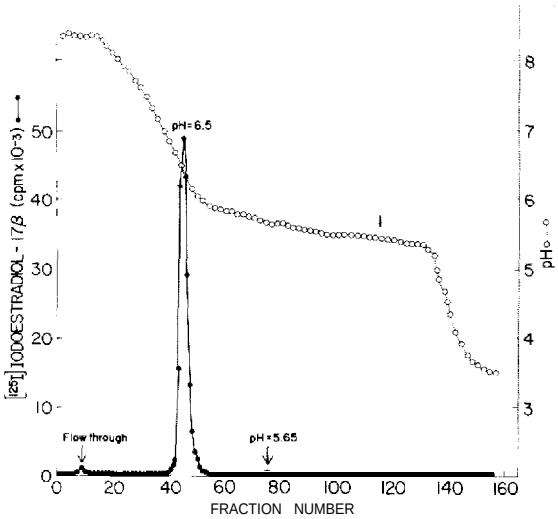

Fig. 10. Interaction of unbound [^{125}I]iodoestradiol-17β with an AX-500 column equilibrated with buffer containing 10 mM sodium molybdate. Steroid was injected into the AX-500 column as a dilute solution in column equilibration buffer containing 10 mM sodium molybdate (see Experimental). Chromatography conditions as described in the legend of Fig. 8.

the one in Fig. 8 were obtained. Although some activity was eluted at pH 6.5, the bulk of the activity was eluted as two distinct peaks at pH 5.0 and pH 3.6. These relatively acidic components appear to be associated specifically with iodoestradiol-17β and represent molybdate-stabilized isoforms of the estrogen receptor which have not been recognized previously. To confirm the receptor nature of these species, non-specific estrogen-binding components were identified by incubation of the molybdate-containing cytosol with [^{125}I]iodoestradiol-17β in the presence of excess radioinert competitor (DES). Chromatofocusing of this non-specific estrogen-binding activity on AX-500 under identical conditions revealed the profile shown in Fig. 9. A profile similar to this was seen when only free [^{125}I]iodoestradiol-17β was chromatofocused with molybdate under identical conditions (Fig. 10). As was the case for the chromatofocusing of free steroid in the absence of molybdate (Fig. 6), a peak of radioactivity eluted near pH 6.5 was observed.

Our data clearly suggest that the large peak of activity eluted at pH 6.5 (e.g. Fig. 7) consists primarily of estrogen-receptor complexes. However, non-receptor components that associate weakly with iodoestradiol-17β as well as unbound steroid are also present. Subsequent analyses of peak activity at this pH by Sephadex G-25 chromatography has indicated that 40–60% of the labeled-steroid was bound to a macromolecule. Thus, the remainder was probably due to free steroid, which dissociated during chromatofocusing.

The two acidic receptor species discovered by rapid chromatofocusing in the presence of molybdate (Fig. 8) appear to be true receptor species. Neither non-specifically-bound steroid nor free steroid was eluted at or near pH 5.0 or pH 3.6. The demonstration of these unique estrogen receptor isoforms in our study with molybdate may be of significance in understanding the interrelations of various types of steroid receptor proteins and the mechanism(s) by which molybdate stabilizes them.

In summary, we have demonstrated that HPLC chromatofocusing is a rapid and highly efficient method for separating estrogen receptor isoforms, varying widely in size and surface charge. The biological significance of our observations regarding microheterogeneity (isoforms) is not fully understood currently. However, the speed and sensitivity of this technique, as well as the high recoveries observed during analyses of impure preparations of estrogen receptors, open a previously unexplored realm of investigation.

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