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ISOFORMS OF ESTROGEN RECEPTORS BY HIGH-PERFORMANCE ION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

SynChropak AX-300, AX-500 and AX-1000 columns were used to separate ionic forms of estrogen receptors by high-performance ion-exchange chromatography. Cytosols from hormone-responsive tissues were incubated for 4–10 h with 3–4 n*M* [16α-^{125}I]iodoestradiol-17β, cleared of unbound ligand and applied to an anion-exchange column. Components were eluted at pH 7.4 using a gradient of phosphate buffer at 4–6°C. Non-specific binding components were identified by chromatographing, the identical cytosol, incubated with [^{125}I]iodoestradiol and a 500-fold excess of diethylstilbestrol, which blocks specific binding sites. [^{125}I]Iodoestradiol was applied to the column in the absence of cytosol and eluted normally to determine the behavior of free ligand. Each column exhibited a different elution pattern for the estrogen receptor. The various isoforms of estrogen receptor were eluted differently from each column usually in the 15–120 m*M* and 180–250 m*M* region of the gradient. Often one non-specific binding component was not retained whereas other non-specific species were retained and eluted from the column in a salt-dependent manner; their position in the gradient varied from column to column. Similarly, free [^{125}I]iodoestradiol was eluted at different positions in the gradients, dependent upon which column was employed. In general, the high flow-rates, reproducibility, good recovery and the apparent differential selectivity of each of the columns appear valuable in the investigation of the nature and subunit composition of the estrogen receptor molecule.

INTRODUCTION

High-performance liquid chromatography (HPLC) has demonstrated its utility in the field of protein purification largely due to the development of spherical, macroporous silica of high mechanical stability as a stationary phase. This material can be surface-modified to yield column packings with properties similar to the plastic gels currently employed in size-exclusion and ion-exchange chromatography. High-performance ion-exchange chromatography (HPIEC) is a rapid and reliable method for the analysis of proteins on the basis of surface charge[1–3].

Our laboratory is concerned with the relationship of the structure of the es-

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trogen receptor and its function in hormone-responsive tissues. We have described multiple forms of the receptor (isoforms) through techniques for protein characterization based upon size, shape and charge[4–7] and have suggested that receptor heterogeneity may be indicative of endocrine responsiveness[4–8]. Many laboratories (including our own) have used open-column anion-exchange chromatography in the analysis of the receptor molecule by taking advantage of its surface charge properties (*e.g.* refs. 9 and 10).

In general, investigations of physical properties of the estrogen receptor have been frustrated by the low levels available in target tissues and their inherent instability under various conditions of analysis. In recent years our laboratory has increasingly employed HPLC methodology to investigate the estrogen receptor on the basis of size and shape by high-performance size-exclusion chromatography (HPSEC)[7], and on the basis of surface charge by high-performance chromatofocusing (HPCF)[6].

This report details our development of HPIEC as a method for the partial purification and characterization of estrogen receptor isoforms from human uterus and breast cancer tissue, rabbit endometrium, and mammary glands from lactating rats. We have compared three high-performance anion-exchange columns (SynChropak AX-300, AX-500, AX-1000) for their ability to separate ionic forms of the estrogen receptor. We have compared these results with those obtained with conventional DEAE-cellulose chromatography. Certain features of these columns appear to provide novel and unique information on isoform distribution and their ligand binding domains.

MATERIALS AND METHODS

Materials

[^{3}H]Water and [16α-^{125}I]iodoestradiol-17β (1800 2000 Ci/mmole) were obtained from New England Nuclear. Diethylstilbestrol (DES) was obtained from Sigma as were mono- and dibasic potassium phosphate, EDTA, glycerol, Norit A (neutral) charcoal, dextran and dithiothreitol (DTT). DEAE-cellulose (DE-52) was obtained from Whatman.

Cytosol preparation and ligand binding reactions

Human breast cancer tissue and uteri were obtained through the aegis of pathologists at local hospitals. Partial purification of the estrogen receptor from human uterus was performed according to Hutchens *et al.*[11]. Samples of endometrium from aged rabbits were obtained from Dr. Jeffrey Nisker. The mammary gland of lactating rat was obtained from animals bred and maintained in our vivarium. In each case, fresh tissue samples were quickly frozen in liquid nitrogen and held at −86°C. Human breast cancer and rodent mammary gland tissues were pulverized at −70°C before cytosol preparation. All subsequent steps were performed at 0–4°C. Tissue samples were homogenized in a Brinkman Polytron using P_{10}EDG buffer (10 m*M* potassium phosphate (pH 7.4) at 4°C, 1.5 m*M* EDTA, 1.0 m*M* DTT, 10% glycerol) in a ratio of *ca.* 1:4 (w/v). The homogenate was centrifuged at 105000 *g* for 30 min to sediment organelles and cellular debris. The cytosol was used immediately in binding reactions by the addition of sufficient [^{125}I]iodoestradiol-17β to give a

final ligand concentration of 3–4 n*M*, which represents a concentration sufficient to saturate estrogen binding sites[4,5]. Parallel incubations were performed in the presence of radio-labeled ligand and 1.5 μM DES. Incubations were terminated after 4–10 h by the addition of an aliquot of incubate to a pellet of dextran-coated charcoal (DCC) obtained from an equal volume of DCC suspension (1.0% charcoal, 0.05% dextran in 15 m*M* Tris–HCl buffer, pH 8.0 at 4°C). Protein was determined by the method of Waddell[12].

High-performance ion-exchange chromatography

A portion (150–200 μl) of incubate cleared of unbound ligand was applied to a Altex 322 (Beckman, Berkeley, CA, U.S.A.) chromatograph equipped with AX-300, AX-500 or AX-1000 (SynChrom) anion-exchange columns. Chromatography was performed in the cold room at 0–6°C. Each column was equilibrated previously with P_{10}EDG. Washing (30 min) of the column with P_{10}EDG was followed by elution with a linear gradient of potassium phosphate at pH 7.4 which approached 500 m*M* 90 min after gradient initiation. Subsequently the column was returned to starting conditions, *i.e.* P_{10}EDG. All buffers were filtered prior to use with a 0.45-μm Millipore filter. The levels of species absorbing at 280 nm were detected as they emerged from the column by passing the eluate through a Hitachi 100-40 spectrophotometer equipped with a low-volume flow cell. Fractions (*ca.* 1 ml) of the eluate were collected and counted for [^{125}I]iodoestradiol-17β using a Micromedics gamma counter with a 62% counting efficiency. The phosphate concentration was determined by measuring the conductivity of fractions and the conductivity of standard phosphate solutions (in glycerol).

Open-column ion-exchange chromatography on DE-52

A portion (150 μl) of incubate was cleared of unbound ligand and applied to a previously washed and equilibrated DE-52 column (25 × 12 mm I.D.). The column was washed briefly with P_{10}EDG then eluted with 160 ml gradient of potassium phosphate at pH 7.4 which approached 500 m*M*. Fractions (*ca.* 1 ml) were counted for radio ligand and their salt concentration determined as described above. Chromatography was performed in the cold room at 0–6°C.

RESULTS AND DISCUSSION

HPIEC separation of estrogen receptor isoforms in human breast cancer using AX-300

The data in Fig. 1 illustrate an HPIEC separation of ionic forms of [^{125}I]iodoestradiol–receptor complexes from human breast cancer on the AX-300 column using a gradient of 10–500 m*M* of potassium phosphate at pH 7.4 in 1.5 m*M* EDTA, 1 m*M* DTT and 10% glycerol. The continuous line represents the profile of material absorbing at 280 nm in the eluent as it emerges from the column; two major regions were identified, namely in the fractions 2–6 and in the fractions 34–50. In general, the early peaks are indicative of proteins which do not interact with the column on the basis of their surface charge, although on these anion exchangers both the porosity and the hydrophobicity of the stationary phase may influence retention characteristics as well[3]. The degeneracy of the early peaks could be indicative of an exclusion effect resulting from its relatively small pore size (300 Å). The second group

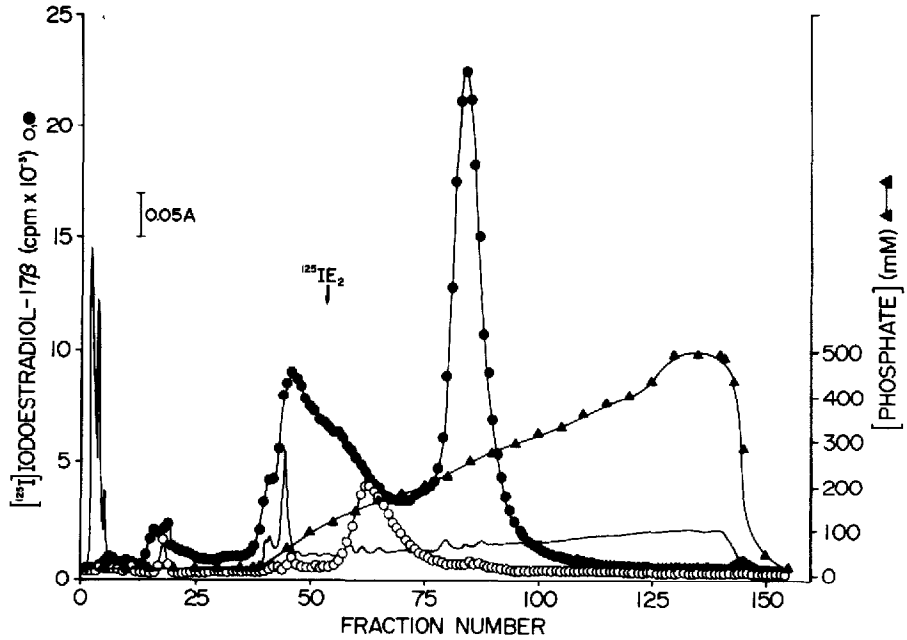

Fig. 1. HPIEC separation of ionic forms of the estrogen receptors from human breast cancer tissue on AX-300. Cytosol was prepared from human breast cancer tissue and incubated in the presence (○) or absence (●) of 500-fold excess DES as described under Materials and methods. Elution was performed at 1.0 ml/min using a gradient of potassium phosphate (▲) at pH 7.4. The elution of the labeled ligand alone was determined previously under identical conditions and is marked with an arrow. The recovery of total radioactivity from the column was 94% for the aliquot of cytosol incubation in the absence of DES. A total of 1.54 mg of protein was applied in 200 μl. A tracing of species absorbing at 280 nm is given by the continuous line.

of species emerging from the column would seem to be eluted on the basis of surface charge since they appear immediately following the initiation of the salt gradient. Additional chromatographic experiments in which the point of initiation of the gradient was altered showed that the elution of these species was salt-dependent and not volume-dependent (data not shown). The rising baseline which is evident between fractions 40 and 140 is due to a difference in absorption between the two buffers used to form the salt gradient. Extensive purification of the isoforms separated by this step was apparent.

Our previous work with these anion-exchange columns in the chromatofocusing mode indicated that [^{125}I]iodoestradiol-17β was selectively retained[6], presumably through a hydrophobic interaction with the stationary phase. We evaluated the elution of unbound [^{125}I]iodoestradiol from the AX-300 column under the ion-exchange conditions. Radioligand was applied to the column in buffer and in the absence of receptor or other proteins. The AX-300 typically returned 90–100% of applied radioactivity and invariably retained the ligand as a single sharp peak at the position shown in Fig. 1. In contrast to the elution of unbound [^{125}I]iodoestradiol-17β, [^{3}H]water was eluted in fraction 4 5 with a high recovery of applied radioactivity (greater than 95%).

A number of [^{125}I]iodoestradiol-binding species are evident from Fig. 1. Their authenticity as receptor species may be inferred by comparing the profiles obtained from chromatography of the same cytosol previously incubated in the presence and absence of competitor. Estrogenic compounds, such as DES, compete for high-affinity, low-capacity sites (receptors), but not for low-affinity, high-capacity sites (non-specific). At least three different groups of estrogen receptor isoforms are evident, namely (1) components which are eluted after the void volume but before the salt gradient (fractions 5–20), (2) components which are eluted in the beginning of the salt gradient (fractions 40–65), and (3) components which are eluted by relatively high salt concentrations (fractions 75–95). For convenience we term these species the very-low-salt (VLS), low-salt (LS), and high-salt (HS) components.

The species eluted in the VLS region of the gradient appear to be heterogenous, containing both specific and non-specific binding components. These components comprise less than 4% of total recovered activity. The VLS components may be either aggregates or minor isoforms which have been enzymatically degraded. They were not eluted with the unretained proteins but appear to exhibit a non-salt-dependent interaction with the column since they were eluted in the isocratic portion of the procedure, *i.e.* at 10 m*M* phosphate buffer. These components were also not eluted with the free [^{125}I]iodoestradiol-17β, indicating that the ligand is protein-bound.

The LS components appear to exhibit heterogeneity. The major species

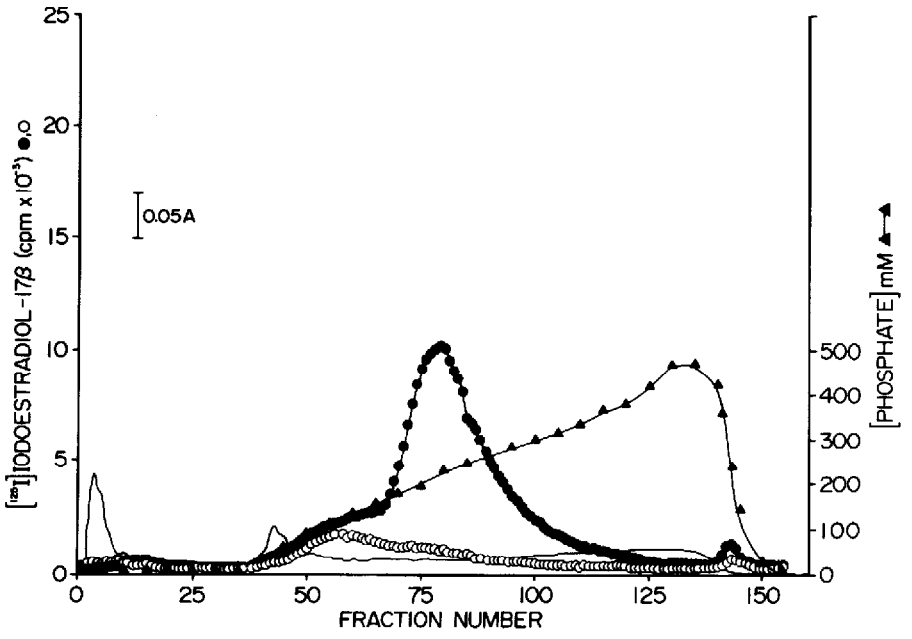

Fig. 2. HPIEC separation of ionic forms of the estrogen receptor from human breast cancer tissue on AX-500. Cytosol identical with that used in the experiment illustrated in Fig. 1 was used. The cytosol was prepared in the presence (○) or absence (●) of 500-fold excess DES. Elution of species from the column was performed at 1.0 ml/min using a gradient of potassium phosphate (▲) at pH 7.4. The recovery of radioactivity from the column was 58% for the aliquot of cytosol incubated in the absence of DES. A tracing of species absorbing at 280 nm is given by the continuous line.

emerged from the column in fraction 41 (65 m*M* phosphate). The broad LS peak exhibited a reproducible shoulder which eluted at 117 m*M* phosphate. The entire LS components represents 36% of the total specific binding activity recovered from the column.

The HS component is eluted as a highly symmetrical peak and appears to be a single ionic species. This moiety emerged from the column with 245 m*M* potassium phosphate. This area represents 54% of the total specific binding activity recovered from the column.

Since unbound ligand was eluted in the same region as the LS components, the interpretation of the resulting ion-exchange profiles could be complicated by spurious counts. However, since the cytosols are always cleared of unbound ligand (with DCC) immediately *prior* to application, there should be little contribution from free ligand. The lack of any appreciable amount of ligand, bound or unbound, appearing in the region for the DES-treated cytosol (Fig. 1), demonstrates the efficiency of sample clearance by DCC. Moreover, the rapid analysis afforded by the use of HPLC technology should minimize the extent of ligand arising by dissociation of the estrogen receptor complex. At the same time, since we cannot rule out the possibility of column-generated denaturation from the estrogen-receptor complex or dissociation of ligand, the retention of unbound [^{125}I]iodoestradiol represents a possible objection or, at least, a less than optimal situation.

HPIEC separation of estrogen receptors isoforms from human breast cancer using AX-500

The data given in Fig. 2 illustrate the separation of ionic forms of the estrogen receptor on the AX-500 column. In these chromatographic separations a cytosol was used which was essentially identical with the one used for the experiments shown in Fig. 1. All parameters of the separation were identical except that in this case a AX-500 column was used.

The most obvious difference between the data given here and in Fig. 1 is the number of specific forms of the estrogen receptor resolved. Fig. 2 shows essentially one ionic form of the receptor which is eluted from the column at 223 m*M* phosphate. The area of the peak represents 37% of the total amount of radioactivity applied, although an identical amount of radioactivity was applied. The overall recovery of this column was 58% in contrast to 94% for the AX-300. Unlike the experimental result given in Fig. 1, there were no peaks of specific binding in the VLS nor, very remarkably, in LS regions. Non-specific binding components were eluted at a salt concentration of 117 m*M* phosphate.

When free ligand was applied to the column and eluted in a way analogous to that described above for the AX-300, the overall recovery was lower and the ligand was eluted in a bimodal fashion, *i.e.* as (a) a broad diffuse peak between fractions 60 and 90 and (b) a second peak after fraction 140. Moreover, the recovery of applied material was lower, apparently owing to the slow leaching of ligand from the column in the second peak. As a result, the regeneration time of the column had to be extended to return to an acceptable background of radioactivity.

These results suggest that the column strongly retained the lipophilic iodoestradiol molecule. A hydrophobic interaction between the steroid ligand and the column support materials could explain the bimodal elution pattern. An increasing salt

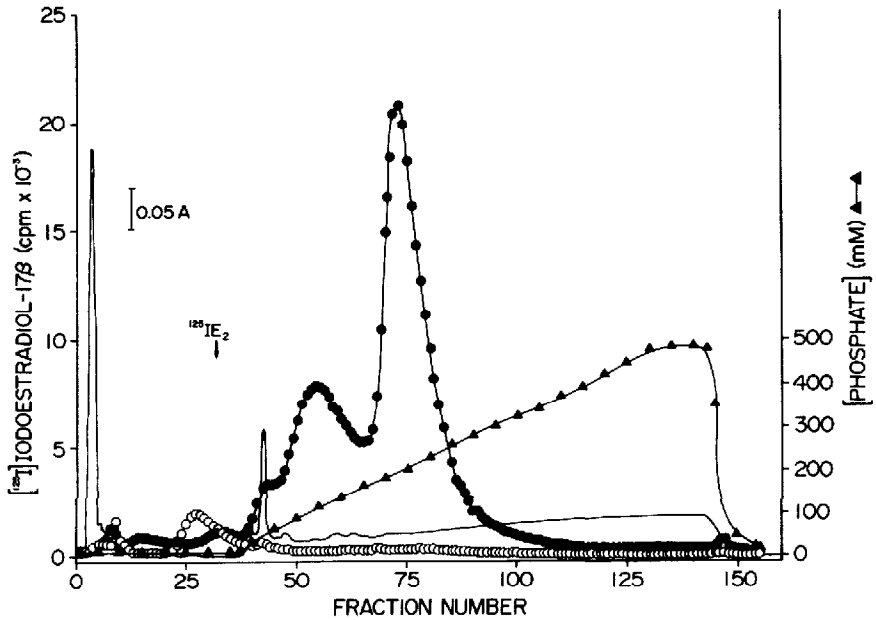

Fig. 3. HPIEC separation of ionic forms of the estrogen receptor from human breast cancer tissue on AX-1000. Cytosol identical with that used in the experiments illustrated in Figs. 1 and 2 was used. The cytosol was prepared and incubated in the presence (○) or absence (●) of 500-fold excess DES. Elution was performed at 1.0 ml/min using a gradient of potassium phosphate (▲) at pH 7.4. The elution of the labeled ligand alone was previously determined under identical conditions and is marked with an arrow. The recovery of radioactivity from the column was 91% for the aliquot of cytosol incubated in the absence of DES. A tracing of species absorbing at 280 nm is given by the continuous line.

gradient could have served to increase the strength of the hydrophobic interactions between the free ligand and the stationary phase resulting in a broad and poorly resolved peak throughout a wide range of salt concentration. The falling salt concentrations after fraction 140 (necessary for column regeneration) could then allow the column-associated ligand to be washed away as a second peak, since in lower salt medium the hydrophobic interactions would be decreased. In contrast, [^{3}H]water was eluted from the column in fraction 4 with an overall recovery of 89%.

The lower recovery of receptor-bound ligand from the column could be attributed to at least two factors, namely (a) a retention of receptor molecule on the column or (b) a loss of ligand or "stripping" of the ligand from the receptor. An answer to these questions may be provided through the use of affinity labeled ligands, post-column labeling, and/or monoclonal antibodies to the receptor molecule. If the column is indeed artifactually stripping ligand, then the total loss of the VLS and LS components in the face of considerably less loss of HS components would imply that the HS components bind estrogen more tightly.

HPIEC separation of estrogen receptor isoforms from human breast cancer tissue using AX-1000

The data given in Fig. 3 illustrate the separation of ionic forms of the estrogen

receptor on the AX-1000 column. These results were obtained from the same cytosol preparation and experimental parameters as those given in Figs. 1 and 2.

The trace of materials absorbing at 280 nm eluted from the column shows the same two major classes of species, *i.e.* an unretained fraction and a group that showed salt-dependent elution. The resolution of components within these two classes by the AX-1000 column was better than by the AX-500 column. The unretained fraction did not exhibit the same degeneracy as in the AX-300 experiment, suggesting that the AX-1000 exchanger with its much larger pore size (1000 Å *vs.* 300 Å) exhibits less tendency to partition protein species on the basis of their ability to penetrate into the pores of the matrix. Concurrently, the similarity between the AX-1000 and AX-300 in terms of the trace of UV-absorbing species in the LS region would suggest that the components in the LS region are not subject to a sizing effect.

The AX-1000 column provided results similar to that given for the AX-300 column and different from the AX-500 column. The major species is the HS component which was eluted at 198 m*M* phosphate. This moiety represented *ca.* 58% of the total specific binding activity. Again the LS region was heterogenous, exhibiting a species eluted at 110 m*M* phosphate and another at 52 m*M* phosphate. This LS species represented 35% of the total specific binding activity applied. The fact that the relative proportions of the two receptor isoforms which comprise this heterogeneous peak are different in the AX-1000 and AX-300 columns could be due to a sieving effect or a hydrophobic effect on ionic exchange processes[3].

One interesting but anomalous result is apparent on Fig. 3. A distinct peak of non-specific binding activity was evident at fractions 23–37. There was no corresponding activity in the sample without competitor. We have no explanation for this finding although we have consistently found that this region exhibits a similar variability for the AX-1000 column for many different tissues and conditions.

This variable region does not appear to be due to unbound ligand. When [^{125}I]iodoestradiol-17β was applied to the column in buffer and in the absence of proteins, it was eluted as a single component with a peak between fractions 29 and 31. As with AX-300 and AX-500 columns, ligand was specifically retained by the AX-1000 system ([^{3}H]water was eluted with the void volume in fraction 4 with 100% recovery). One advantage of AX-1000, however, is that the free ligand can be washed out of column quickly (35 min) and need not interfere with the elution of estrogen receptor isoforms. The combination of low interaction with the steroid, excellent receptor recovery and large matrix pore size indicates the AX-1000 column is the system of choice for the analysis of estrogen receptor isoforms by HPIEC.

Separation of estrogen receptor isoforms from human breast cancer tissue using DEAE-cellulose chromatography

Fig. 4 illustrates the separation of ionic forms of the estrogen receptor from human breast cancer tissue by open-column chromatography on DE-52. The sample applied was the same as that used for the chromatographic experiments performed previously by HPIEC on the AX-300, AX-500 and AX-1000 columns. Buffers and other conditions were identical although the gradient was longer.

At least three major ionic forms of the estrogen receptor were evident (Fig. 4). The first species is eluted in a VLS portion of the gradient. This early eluted species was present in much higher amounts than seen in any of the high-performance an-

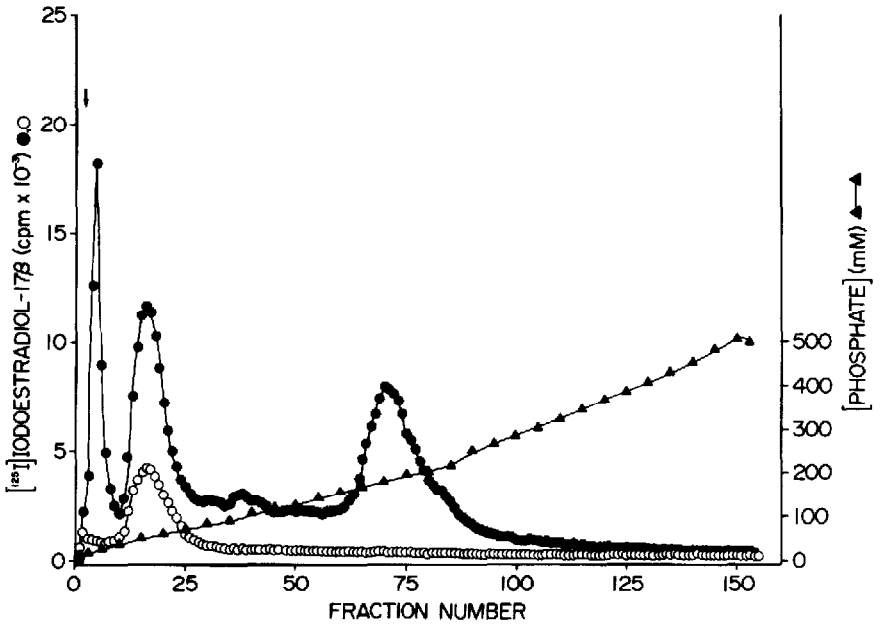

Fig. 4. Open-column chromatographic separation of ionic forms of the estrogen receptor from human breast cancer tissue on DE-52. Cytosol identical with that used in experiments illustrated in Figs. 1–3 was prepared and incubated in the presence (○) or absence (●) of 500-fold excess DES. Elution was performed at *ca.* 1.0 ml/min using a gradient of potassium phosphate at pH 7.4 (▲). The recovery of radioactivity from the column was 62% for the aliquot of cytosol incubated in the absence of DES. The major peak of protein eluted from the column at the point marked by the arrow.

ion-exchange columns and, in fact, it represented nearly 20% of the total specific binding recovered from the column. Based on the high amount of specific activity recovered, this species would not seem to be directly comparable to the (minor) VLS species detected on the AX-300 column although it was eluted in very-low-salt buffer.

The second peak eluted from the column at 55 m*M* phosphate and appeared similar to one of the LS species described previously. A non-specific binding component was eluted with this species. Finally, a species eluted from the column at 180 m*M* phosphate and appeared analogous to the HS species described previously for all three HPIEC columns. The HS species represented 40% of the total specific binding recovered. Largely lacking in the present chromatographic separation was an indication of a distinct peak in the region eluted with 100 120 m*M* potassium phosphate although a significant portion (25%) of the specific binding was recovered in this region. The overall low recovery (62%) of radioactivity from this column further complicated the interpretation.

In general, chromatography of the same receptor preparations on the four different columns indicates certain differences, particularly in the respective LS region of the elution gradients. The AX-500 column showed no species in this portion of the gradient whereas the AX-300 and AX-1000 columns demonstrated apparent heterogeneity of peaks in which the more prominent component was eluted at different salt concentrations dependent upon the column utilized. The DE-52 column resolved two distinct species recovered between 15 and 70 m*M* phosphate. These differences

were not necessarily unexpected since a recent preliminary report suggested that the estrogen receptor from calf uterus can interact in a different manner with different ion-exchange materials[13].

One explanation for the behavior described for the estrogen receptor on different columns involves the possibility that the receptor species recovered in the LS region is appreciably hydrophobic. The differential interaction of [^{125}I]iodoestradiol-17β with each of three high-performance ion-exchange columns suggests that the columns retain lipophilic materials to different extents. Concurrently, the A_{280} profiles of cytosol components on each of the three columns were remarkably similar, indicating that, in general, the vast majority of the protein constituents of the cytosol were eluted in a like fashion on the AX-300, AX-500, and AX-1000 columns, namely, either immediately following sample application (void volume) or immediately following the initiation of the salt gradient. On this basis, the estrogen receptor recovered from the heterogenous LS region on the AX-300 and AX-1000 columns would appear to be considerably more hydrophobic than the majority of other cytosolic proteins.

The minor variability of the estrogen receptor species recovered from the HS regions would also be consistent with our proposal that these estrogen receptors exhibit hydrophobic properties. Thus, care must be taken when comparing the three peaks identified by DE-52 chromatography with peaks on high-performance anion-exchange columns. The data suggest that the HS species are the same on the four

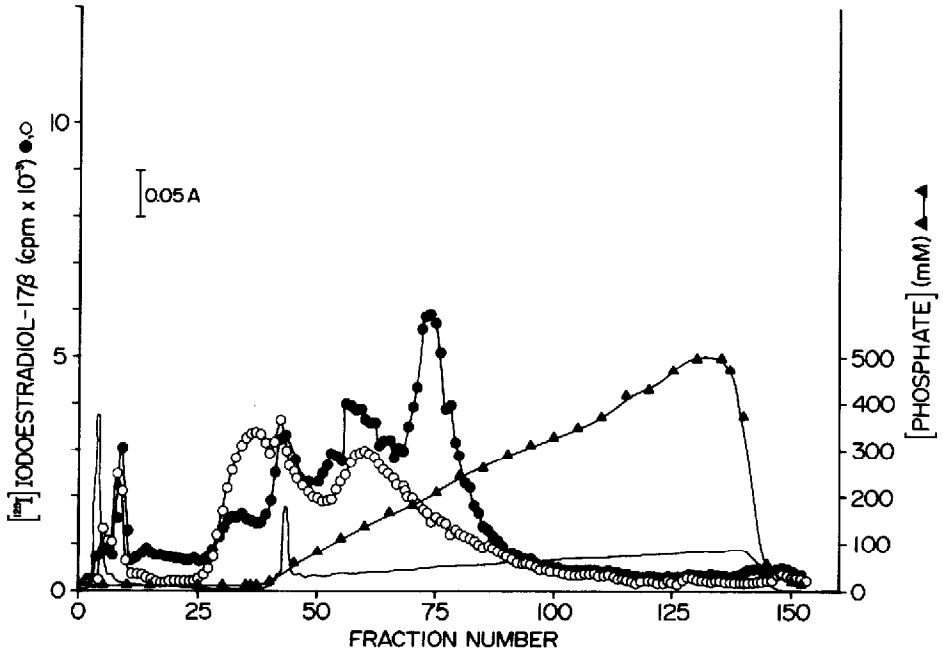

Fig. 5. HPIEC separation of ionic forms of the estrogen receptor from human uterus on AX-1000. Cytosol was prepared from human uterus as described in Methods. The cytosol was prepared and incubated in the presence (○) or absence (●) of 500-fold excess DES. Elution was performed at 1.0 ml/min using a gradient of potassium phosphate at pH 7.4 (▲). A tracing of species which absorb at 280 nm is given by the continuous line.

columns and that the two LS species from the DE-52 chromatography are recovered in the heterogenous LS gradient region of the AX-300 and AX-1000 columns. Clearly, additional experiments involving ion-exchange chromatography and/or reversed-phase chromatography would help to explore these phenomenon.

HPIEC separation of estrogen receptor isoforms from other tissues using AX-1000

In order to assess the validity of HPIEC as a general method of analysis of the estrogen receptor from hormone sensitive tissues, we investigated the ionic forms from a number of species and tissues. Fig. 5 illustrates the separation of ionic forms of the estrogen receptor from human uterus on AX-1000 under the conditions described in Fig. 3. It is evident from Fig. 5 that a similar profile was obtained for the receptor from human uterus as for those from human breast cancer. In each case, a non-specific binding component was eluted after the void volume, and specific estrogen receptor species were eluted at 115 m*M* and 203 m*M* potassium phosphate. Also apparent from Fig. 5 is the high radioactivity recovered in the variable region (fraction 37).

One difference between the data shown in Figs. 3 and 5 concerns a second non-specific component which was eluted at 135 m*M* phosphate. The lack of such a component in the human breast cancer specimen implies this species is tissue-specific.

Table I contains the results of our investigations of the isoforms of the estrogen receptor from a number of tissues and species. In each of the tissues analyzed, the HS component was the major isoform recovered and eluted between 180 and 245 m*M* phosphate. Usually there was another specific binding component eluted in the 80–120 m*M* phosphate range, although its appearance was highly dependent upon the type of column used as well as the tissue under investigation. In general, the AX-1000 column performed better than the other columns in terms of recovery (greater than 90%) and number of forms resolved.

TABLE I

SUMMARY OF IONIC FORMS OF ESTROGEN RECEPTORS SEPARATED BY HPIEC

Tissue	*Estrogen receptor isoforms**		
	AX-300	*AX-500*	*AX-1000*
Human breast cancer	65		53
	117		110
	245	225	190
Human uterus	ND**	ND	115 (47)***
			203
Rabbit endometrium	ND	ND	36
			106
			215
Rat mammary gland	88		90
	204	180	205

* Phosphate concentration (m*M*) which led to elution.

** ND = Not determined.

*** Purified *ca.* 8000-fold (see Fig. 7).

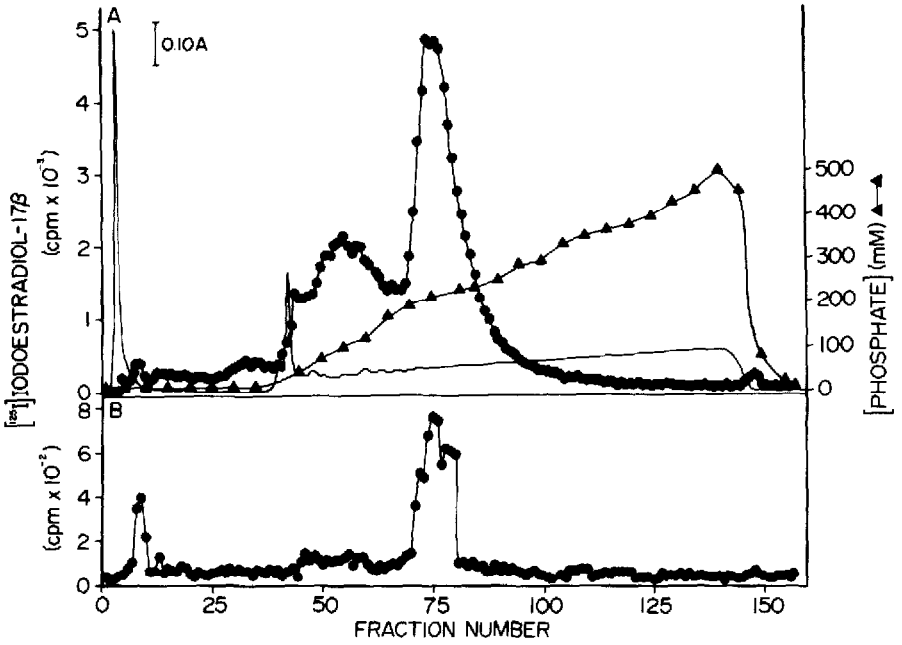

Fig. 6. Effect of DCC treatment on the ionic forms of the estrogen receptor separated by HPIEC. (A) Cytosol identical with that used in the experiments illustrated in Figs. 1–4 was used. Cytosol was incubated for 4 h with 3 n*M* [^{125}I]Iodoestradiol-17β (●). A portion of the incubate was cleared of unbound ligand and applied to an AX-1000 column in 400 μl. Elution was performed at 1.0 ml/min using a gradient of potassium phosphate at pH 7.4 (▲). A 330-μl aliquot of each fraction was removed for counting. (B) A 200-μl aliquot of each fraction was removed to DCC pellets derived from an equal volume of DCC suspension, mixed briefly, then centrifuged for 5 min at 3000 *g* to sediment the charcoal. A 100-μl aliquot of each cleared supernatant was removed for counting (●).

Effect of DCC treatment on the isoforms of the estrogen receptor separated by HPIEC.

In the experiments described above, we have assumed that the radioactivity recovered is largely associated with the binding sites on the receptor or on other proteins. The recovery of free ligand from the column at unique elution positions would indicate this also. However, the apparent variability of the profiles in the LS region may be due to denaturation of the receptor by the column packings and release of free ligand. An additional concern has been the apparent instability of the LS species resulting in the loss of ligand due to increased dissociation or stripping by the AX-500 column.

To test whether the [^{125}I]iodoestradiol-17β recovered from the column was bound to protein and whether the LS and HS species were equally stable after partial purification on HPIEC, the following experiment was conducted. A sample identical with the cytosol separated in the chromatograms described in Figs. 1–4 was applied to the AX-1000 column and eluted. The profile of this separation is given in Fig. 6A. The similarity of the profiles in Figs. 6A and 3 demonstrates the reproducibility of the method. After separation, aliquots of the eluate were counted and others were treated with pellets of DCC to clear the sample of unbound ligand.

As indicated in Fig. 6B, after treatment of the eluate fractions with DCC it

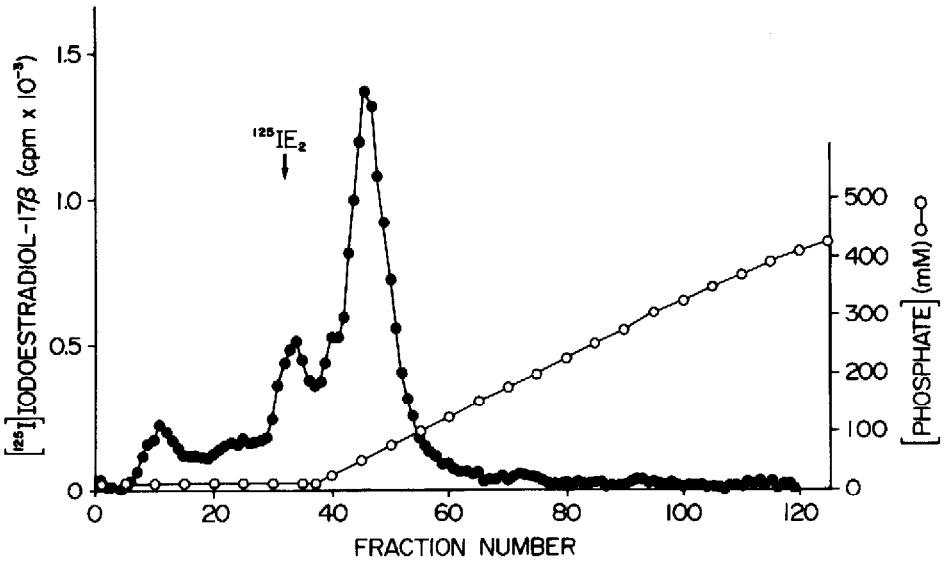

Fig. 7. HPIEC separation of ionic forms of the estrogen receptor from purified human uterus on AX-1000. The estrogen receptor from human uterus was partially purified and allowed to bind [16α-^{125}I]iodoestradiol-17β (●). Unbound ligand was removed and a 500-μl aliquot was applied to the AX-1000 column (250 × 4.1 mm I.D.). Elution was performed at 1.0 ml/min using a gradient of potassium phosphate (○). A tracing of species which absorb at 280 nm is given by the continuous line.

was apparent that the HS fraction was preferentially preserved, although there was loss of activity in the LS region. Extensive loss of ligand in the LS region could be due to enhanced dissociation of this receptor isoform, enhanced susceptibility of this isoform to stripping, or artifactual accumulation of unbound ligand in this region of the gradient when cytosol was separated (in distinction to the control case when only ligand in buffer is run). Partial stripping of the steroid from receptor sites by DCC had been observed before with purified preparations of receptor. The recovery of ligand in the VLS region after DCC treatment indicated that this non-specific binding component was highly resistant to stripping, dissociation or column effects.

To further test the protein-bound nature of the LS component, another aliquot of the eluate from this region was re-applied to the AX-1000 column. The extremely low levels of protein applied in this second separation should have little effect on the profile. Thus, if the radioactivity was unbound, it should have behaved as unbound ligand, whereas if the radioactivity was bound to receptor it should reappear in the LS portion of the gradient. In this instance, the radioactivity was recovered in the same region as before, *i.e.* at 95–110 m*M* phosphate in the LS region and not as unbound ligand (data not shown). On the basis of these experiments, it appears that at least one of the LS and the HS species represent protein-bound species.

HPIEC separation of purified estrogen receptor isoforms in human uterus on AX-1000

To further confirm the authenticity of the LS receptor form, a partially purified preparation of the estrogen receptor from human uterus was separated on the AX-1000 column under the same conditions as described in Figs. 3 and 5. Radioligand

was shown to be associated with protein by several other independent criteria[11]. The chromatographic separation is given in Fig. 7. In this case, the labeled receptor, once partially purified, was recovered at 47 m*M* phosphate as an LS species. This is in contrast to the profile of isoforms in the unpurified preparations (Table I), where the major species of the LS region was recovered at 115 m*M* phosphate. Apparently this third isoform was a minor component in the unpurified uterine preparation which was identified only after extensive purification by affinity chromatography.

In summary, HPIEC may be used to separate ionic forms of the estrogen receptor from a variety of tissues using the SynChropak AX-300, AX-500 and AX-1000 columns. The separation profiles of receptor isoforms are distinct from one another perhaps due to variations in column charge density or hydrophobicity. Since separation profiles of receptor isoforms were similar with either AX-300 or AX-1000, porosity does not appear to be a contributing factor. Additionally the three columns each interact with unbound [^{125}I]iodoestradiol-17β to a different extent. Finally, the profiles were considerably different from those obtained by conventional ion-exchange chromatography. Based on the various approaches outlined earlier, such as removal of unbound ligand by dextran-coated charcoal or rechromatography of individual isoforms, our investigations suggest there are at least three different isoforms of the estrogen receptors in the tissues examined. Of importance to our studies is that the differences between the columns may be exploited to advantage in the determination of the origin of receptor heterogeneity and the interrelationships of the various estrogen receptor isoforms.

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