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HIGH-PERFORMANCE SIZE EXCLUSION CHROMATOGRAPHY AS A RAPID METHOD FOR THE SEPARATION OF STEROID HORMONE RECEPTORS

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SUMMARY

High-performance size exclusion chromatography (HPSEC) on TSK 3000SW molecular sieve columns was used to separate estrogen, progestin, and androgen receptors from several target tissues within 50 min on the basis of size and shape (Stokes radius). Moreover, this system provided for the detection of heterogeneity of receptor species (isoforms) in a manner superior to that observed with sucrose density gradient centrifugation. HPSEC separated various estrogen receptor isoforms having Stokes radii of > 61 Å, ~ 48 Å and 29—32 Å. Agents such as potassium chloride and sodium molybdate which alter the distribution of estrogen receptor species on sucrose density gradient centrifugation, promote similar alterations in receptor profiles when HPSEC was employed. Our investigations suggest the use of [^{125}I]iodoestradiol-17β and HPSEC allows the sequential analysis of estrogen receptor species providing new insights into receptor composition and structure.

It is concluded that HPSEC has a broad application in the field of steroid hormone receptors. This method should be useful in studies ranging from measurements of molecular and kinetic properties to their mode of cellular interaction and regulation.

INTRODUCTION

Intracellular proteins termed receptors exist for the steroid hormones. These constituents appear to be a prerequiste for eliciting a biological response by a target organ [1—3]. Current methodologies for the analyses of these proteins (gel chromatography, isoelectric focusing, ion-exchange chromatography, sucrose density gradient centrifugation) are laborious and time-consuming considering the highly labile nature of these macromolecules [3]. For example, the

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variety of molecular forms of the estrogen receptor has been attributed to such diverse causes as subunit dissociation, enzymatic processing, and proteolysis. Recently we described a rapid and efficient procedure for resolution of these forms on the basis their surface charge using either high-performance chromatofocusing [4] or high-performance ion-exchange chromatography [5].

In this study we explored rigid, macroporous size exclusion columns for protein separation in a high-performance size exclusion chromatographic (HPSEC) system similar to those described by Regnier and Gooding [6] as a means of separating various species (isoforms) of a given class of steroid hormone receptors on the basis of size (i.e. Stokes radius). The speed and efficiency of this technology make it ideal for investigating molecular properties of steroid hormone receptors. These data on size and shape may be compared with those obtained from gel chromatography and sucrose density gradient centrifugation to determine the interrelationships of the receptor isoforms.

It was of particular interest to determine if HPSEC was as useful for the characterization of estrogen receptors as sucrose density gradient centrifugation. The latter procedure is utilized currently as a clinical method of determining the molecular species of estrogen receptors in human breast cancer [7, 8]. To determine if our earlier proposal regarding molecular heterogeneity is viable [9, 10] the influence of a number of conditions such as ionic strength, buffer type, pH and protease inhibition were evaluated. Also the clinical utility of the HPSEC method has been examined using radioactively labeled ligands for estrogen, progestin, and androgen receptors in a variety of tissues.

In human breast carcinoma, the concentration [11] and properties [9, 12] of estrogen receptors are valuable biochemical criteria for selecting hormonal therapies by the clinician. Our data clearly indicate that the use of size exclusion columns in high-performance liquid chromatography (HPLC) provides rapid separation of steroid hormone receptors which may be applied as a clinical method. This may be particularly significant with metastatic breast carcinoma where biopsies usually are smaller. In this case, we suggest that ^{125}I-labeled estradiol-17β be used as the ligand due to its high-specific radioactivity [10, 13].

MATERIALS AND METHODS

Reagents and chemicals

The ligands used for receptor studies, [16α-^{125}I]iodoestradiol-17β, [2,4,6,7-^{3}H]estradiol-17β, [17α-*methyl*-^{3}H]methyltrienolone (^{3}H-R1881), [11β-*methoxy*-^{3}H]moxestrol (^{3}H-R2858), [17α-*methyl*-^{3}H]promegestrone (^{3}H-R5020), and several of their unlabeled counterparts were obtained from the New England Nuclear Corporation. Purity of labeled steroids was checked by thin-layer chromatography using two solvent systems; only those with purities of $> 95\%$ were utilized. Other unlabeled ligands such as diethylstilbestrol were purchased from Sigma.

The purified proteins used as markers, namely horse cytochrome *c*, chicken ovalbumin, whale myoglobin and hemoglobin, catalase, and thyroglobulin of human origin were obtained from Sigma.

Animals and tissues

Lactating mammary glands of Sprague-Dawley rats were obtained from animals bred in our vivarium. Calf uterus was obtained from animals at the time of sacrifice in a local slaughterhouse. All human tissues were obtained through the aegis of pathologists at local hospitals where tumor pathology was confirmed. Specimens were either frozen in pathology or brought to the laboratory on ice and frozen in liquid nitrogen. Usually human breast carcinomas were used in the form of a powder consisting of pooled specimens pulverized in liquid nitrogen and stored at −86° C.

Preparation of cytosol and ligand-binding reactions

Fresh tissue was minced at 0—4° C whereas frozen tissue was sliced into thin sections with a scalpel blade before mincing. Pulverized tissue was mixed directly with buffer prior to homogenization. Homogenization was carried out at 0—4° C utilizing a Brinkman Polytron [3, 4]. One of the following homogenization buffers was used: TEGM [10 m*M* Tris—HCl, pH 7.4 at 4° C, containing 1.5 m*M* EDTA, 10 m*M* monothioglycerol, 10% (v/v) glycerol]; PEGM [25 m*M* Na_2HPO_4/NaH_2PO_4, pH 7.4 at 4° C, containing 1.5 m*M* EDTA, 10 m*M* monothioglycerol, 10% (v/v) glycerol]; TEGMM [10 m*M* Tris—HCl, pH 7.4 at 4° C, containing 1.5 m*M* EDTA, 10 m*M* monothioglycerol, 10% (v/v) glycerol, 20 m*M* sodium molybdate]. Homogenates were centrifuged at 105,000 *g* for 30 min to sediment nuclei, organelles, and cellular debris. The infranates were separated from the lipid-rich layer and used immediately in binding studies. The cytosolic protein concentration was determined by the method of Waddell [14] using bovine serum albumin as a standard. When cytosols prepared in the presence of molybdate were assayed, the Waddell method was modified since molybdate interferes with this procedure. In this case, the dual-beam spectrophotometer was used and initially balanced using molybdate-containing buffers diluted to the same extent as the unknowns (1:100). Protein standards were prepared in molybdate-containing buffer, read at the appropriate wavelengths and the standard curve constructed. These curves were linear although the parameters of the line were different from the non-molybdate-containing standard curves.

All ligand binding reactions were carried out at 0—4° C. Cytosols were incubated with a saturating concentration (4—5 n*M*) of one of the labeled ligands either in the presence or absence of a 200- to 250-fold excess of unlabeled steroid or competitor to estimate non-specific (low-affinity, high-capacity) binding [3]. Certain incubations were performed in 400 m*M* potassium chloride to achieve a high ionic strength. The reactions were terminated after various times by adding the reaction mixture to pellets of dextran-coated charcoal which removed unbound ligand from solution [4]. A low-speed centrifugation step precipitated the charcoal.

Sucrose density gradient centrifugation

Linear gradients of 10—35% (w/w) sucrose were made by hand layering solutions of different sucrose concentrations into $\frac{7}{16}'' \times 2\frac{3}{8}''$ Beckman cellulose nitrate centrifuge tubes. The original sucrose solutions were made using various buffers: TEK (10 m*M* Tris—HCl, pH 7.4 at 4° C, containing 1.5 m*M*

EDTA and 400 m*M* KCl); TE (10 m*M* Tris—HCl, pH 7.4, containing 1.5 m*M* EDTA) or PE (25 m*M* Na_2HPO_4/NaH_2PO_4, pH 7.4, containing 1.5 m*M* EDTA).

The binding reactions were applied to gradients as a narrow band of 200 μl. Marker proteins such as catalase may be applied at this time in a small volume (20—50 μl) to the gradient. The gradients were centrifuged at 60 000 rpm (335 000 *g*) for 16.5 h at 0—4°C ($\omega^2 t = 2.25 \times 10^{12}$). The position of the heme-containing proteins in the fractionated gradients was visualized by taking advantage of their ability to react and form a colored species after the additions of 1% benzidine in glacial acetic acid and 3% hydrogen peroxide. Other protein markers were identified spectrophotometrically.

Calculation of specific binding capacity

Gradient or chromatography fractions containing tritium-labeled steroid were counted in a Beckman LS-9000 scintillation counter. [^{125}I] Iodoestradiol was counted in a Beckman 4000 gamma counter giving 65% counting efficiency. Specific-binding capacity was expressed as fmol/mg cytosol protein using the difference betweeen the accumulated total binding and the binding in the presence of excess unlabeled ligand.

High-performance size exclusion chromatography

All chromatography was performed in a cold room (0—5°C) utilizing the Spherogel TSK-3000SW size exclusion column (7.5 × 700 mm) with a Beckman Model 322 HPLC system equipped with an in-line Hitachi Model 100-40 spectrophotometer. The chromatographic column comprised two separable units, a short (7.5 × 100 mm) TSK 3000SW guard column and, immediately downstream, the longer (7.5 × 600 mm) TSK 3000SW size exclusion column. Reactions were applied in 20—250 μl volumes using a Hamilton syringe and the Model 210 sample injection valve. Essentially no difference in resolution was noted when injection volumes of 20—250 μl and protein concentrations of 2—12 mg/ml were used. An additional aliquot was taken at this time to estimate specific binding capacity and recovery. The elution buffers were either $TEGK_{100}$ [10 m*M* Tris—HCl, pH 7.4 at 4°C, containing 1.5 m*M* EDTA, 10% (v/v) glycerol, 100 m*M* KCl], $TEGK_{400}$ [10 m*M* Tris—HCl, pH 7.4 at 4°C, 1.5 m*M* EDTA, 10% (v/v) glycerol, 400 m*M* KCl] or $PEGK_{100}$ [25 m*M* NaH_2PO_4/Na_2HPO_4 buffer, pH 7.4 at 4°C, containing 1.5 m*M* EDTA, 10% (v/v) glycerol, 100 m*M* KCl]. The $PEGK_{100}$ and $TEGK_{100}$ buffers gave similar results on the HPSEC when used for incubation and elution. All buffers were filtered with a 0.45-μm filter (Millipore). Elution was carried out at a flow-rate of 0.7 ml/min. Column effluent was collected as either 0.5- or 1-min fractions. Following a day of chromatography, the entire column was washed overnight with filtered, distilled, deionized water. The entire column was washed weekly with a filtered solution of 15% dimethyl sulphoxide in methanol whereas the TSK 3000SW guard column was washed periodically with a solution of 6 *M* urea. The chromatographic system was stored in filtered, distilled, deionized water.

RESULTS AND DISCUSSION

Comparison of HPSEC and sucrose density gradient centrifugation

Our early experiments compared the size exclusion properties of estrogen receptors in a variety of hormone target tissues. The lactating mammary gland of the rat is known to contain predominantly a single form of the estrogen receptor which sediments at 8—9 S using buffers containing Tris [15]. An example of these receptors in cytosol of mammary gland from a rat, 15 days post partum, is shown in Fig. 1A. These receptors were extracted in PEGM buffer as described in Materials and methods.

When an identical sample of cytosol was applied to a TSK-3000SW column and eluted with $PEGK_{100}$ buffer, the profile shown in Fig. 1B was observed. The specific estrogen binding capacity was distributed primarily as a high-molecular-weight species eluting just after the void volume of the column seemingly analogous to the 8—9 S form observed with the sucrose gradient method. Unbound [^{125}I]iodoestradiol-17β eluted at the total volume of the column, i.e. at approximately 41 min. It has been our experience that the amount of unbound ligand remaining after dextran-coated charcoal treatment

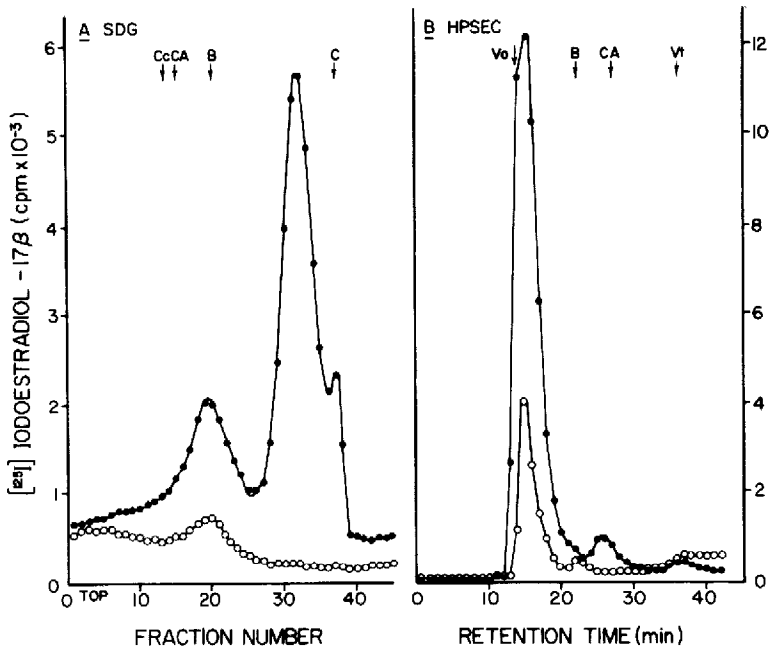

Fig. 1. Comparison of HPSEC and sucrose gradient centrifugation (SDG) for the separation of estrogen receptors from rat lactating mammary gland. Mammary glands of rats lactating 15 days were used as a tissue source of cytosol. Incubation was performed as described in Materials and methods using PEGM buffer and 4 n*M* [16α-^{125}I]iodoestradiol-17β as ligand in the presence (○) or absence (●) of a 250-fold excess of diethylstilbestrol. A portion of each reaction was used for analysis either by sucrose density gradient centrifugation (A) or by HPSEC (B). (A) A 200-μl aliquot was loaded onto a linear gradient of sucrose (10—35%) in PE buffer and centrifuged as described in Materials and methods. (B) A 25-μl aliquot was applied to the TSK 3000SW column and the receptors were eluted with $PEGK_{100}$ buffer as described in Materials and methods. Cc = cytochrome *c*; CA = carbonic anhydrase; B = bovine serum albumin; C = catalase; Vo = void volume as determined using blue dextran; Vt = total volume as determined using ^3H_2O.

and eluting in this position is highly variable and, in certain cases, seems to leach slowly from the column representing a potential problem in terms of interpretation of data. The high non-specific binding peak present at the void volume (Fig. 1B) of the column is a finding representative of cytosol from lactating mammary gland which is not observed when separated on sucrose gradients. This non-specifically associated ligand may represent an artifact in which the components were preferentially preserved by the HPSEC method in distinction to the sucrose gradient procedure.

A similar comparison of separation profiles by sucrose gradient centrifugation and HPSEC was conducted using estrogen receptors from human breast carcinomas [10]. Earlier we reported that breast tumors exhibited a wide variety of estrogen receptor profiles including those that contain both the high- (8—9 S) and low- (4—5 S) molecular-weight forms, only one or the other species or neither [3, 9, 10]. HPSEC revealed three estrogen binding components including a species of large size which eluted from the column near the void volume [10].

In certain experiments in this study using human breast tumors, there was good agreement on the distribution of receptor isoforms using HPSEC and sucrose density gradient centrifugation with the exception of the high-molecular-weight species. The recovery of specific estrogen binding capacity usually was comparable (75—98%) for the two methods although this was highly dependent upon the characteristics of the individual columns.

HPSEC separation of estrogen receptors in uterus

To demonstrate the utility of HPSEC methodology in the analysis of steroid receptors, we examined these proteins in a wide variety of estrogen responsive tissues. Using calf and human uteri, estrogen receptors were extracted in PEGM

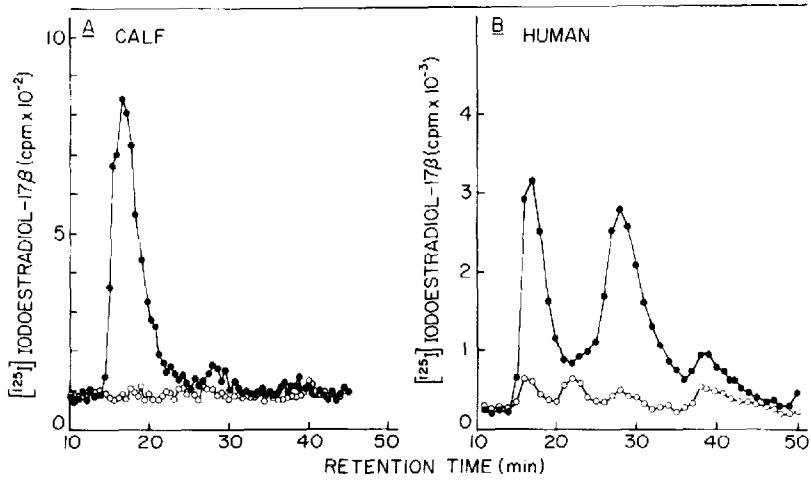

Fig. 2. HPSEC of estrogen receptors from uterus. Calf uterus (A) or human uterus (B) was used as a tissue source of cytosolic estrogen receptors. Cytosol was prepared and incubation performed as described in Materials and methods using PEGM buffer and 5 n*M* [16α-^{125}I]-iodoestradiol-17β as ligand in the absence (•) or presence (○) of a 250-fold excess of diethylstilbestrol. A 200-μl aliquot of each reaction was applied to the TSK 3000SW column. The column was eluted using $PEGK_{100}$ buffer at a flow-rate of 0.7 ml/min.

buffer and separated on TSK 3000SW columns (Fig. 2). As shown, cytosol from calf uterus (Fig. 2A) exhibited a single species which had a Stokes radius of > 61 Å. Human uterus consistently exhibited both a high-molecular-weight form (> 61 Å) and a species with Stokes radius of 29—32 Å (Fig. 2B). We have observed isoforms of the estrogen receptor in human endometrial carcinoma and leiomyomata also [16].

HPSEC separation of receptors for progestins and androgens

The size exclusion properties of progestin receptors in myometrium, ovary and breast carcinoma were estimated using TSK 3000SW columns (Fig. 3). Surprisingly the profiles contained a single receptor species of high molecular weight. Using marker proteins, the estimated size of this binding component was > 61 Å (Stokes radius) similar to that observed for the estrogen receptor. Lower-molecular-weight species were observed rarely, perhaps due to the rapid separation afforded by the HPSEC.

A human ovarian follicular cyst was used as a source of androgen receptor for separation by HPSEC (Fig. 4). The synthetic androgenic ligand, R1881, was used to detect these receptors. Again a high-molcular-weight species (Stokes radius > 61 Å) of the receptor was demonstrated. Unlike most tissues examined, this preparation exhibited a large peak of unbound ligand (fractions 38—42).

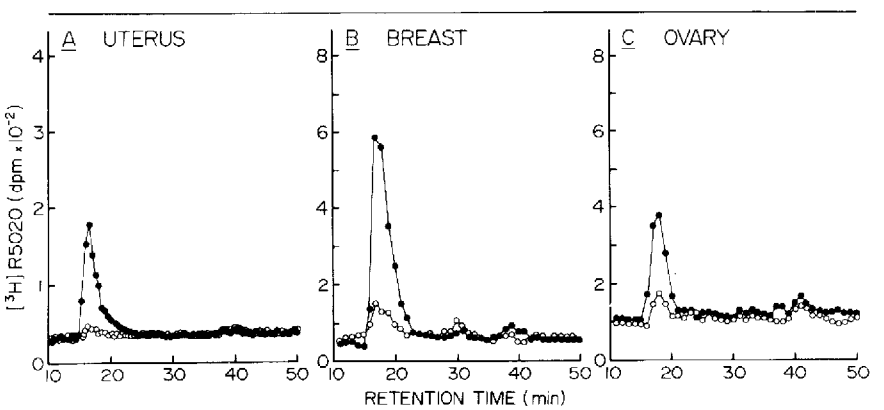

Fig. 3. HPSEC of progestin receptors from various human tissues. Human myometrium (A), human breast carcinoma (B), and human ovary (C) were used as tissue sources of the cytosolic progestin receptors. Cytosols were prepared and the reactions were performed as described in Materials and methods using PEGM buffer and 4 n*M* ^{3}H-R5020, a synthetic progestin, as ligand in the presence (○) or absence (●) of a 200-fold excess of unlabeled R5020. A 250-µl aliquot of reach reaction was applied to a TSK 3000SW column and the receptors were eluted with $PEGK_{100}$ buffer at a flow-rate of 0.7 ml/min.

Influence of potassium chloride and sodium molybdate on separation properties

As discussed earlier, estrogen receptors prepared in low-ionic-strength buffers containing monothioglycerol separated primarily as 8—9 S and 4—5 S components on linear sucrose gradients subjected to centrifugation (Figs. 1A and 5A). In a fashion similar to that which we reported earlier for lactating mammary gland [15], the 8—9 S component in cytosol of human breast

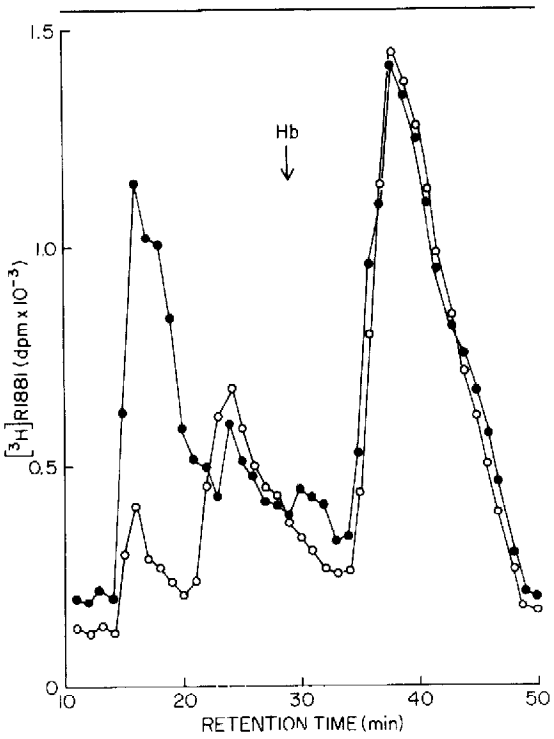

Fig. 4. HPSEC of androgen receptors from human ovarian follicular cyst. A human ovarian follicular cyst was used as a tissue source of the cytosolic androgen receptors. Cytosols were prepared and the reactions were performed as described in Materials and methods using PEGM buffer and ^{3}H-R1881 as ligand in the presence (○) or absence (●) of a 200-fold excess of unlabeled R1881. A 250-μl aliquot of each was applied to a TSK 3000SW column and the receptors were eluted with $PEGK_{100}$ buffer at a flow-rate of 0.7 ml/min. Hb = hemoglobin.

cancer appeared to dissociate into 4—5 S forms in the presence of 400 m*M* potassium chloride (Fig. 5B). However if 20 m*M* sodium molybdate was added to the homogenizing buffer and the receptors separated on linear sucrose gradients, an increased quantity of the 8—9 S species of the estrogen receptor was observed (Fig. 5C). These findings are consistent with the results of a number of investigators using a variety of receptors [17—20].

A problem of considerable magnitude in discerning the interrelationships of estrogen receptor species has been the long separation times required by the sucrose gradient centrifugation which favor both ligand and subunit dissociation and/or receptor degradation. HPSEC was employed to assess the relative importance of these problems since it may be an alternative method. Fig. 6A illustrates the separation of estrogen receptor species in human breast cancer on HPSEC. These profiles were obtained using the same cytosols as those utilized in the sucrose gradient profiles illustrated in Fig. 5. Each HPSEC separation was accomplished within 1 h while centrifugation required 16 h.

Fig. 6A is a representative profile of estrogen receptors in human breast cancer extracted in TEGM buffer and separated by HPSEC in the presence of $TEGK_{100}$ buffer. It is our experience that this is the minimal ionic strength (100 m*M*) required to maintain a linear relationship in the elution sequence

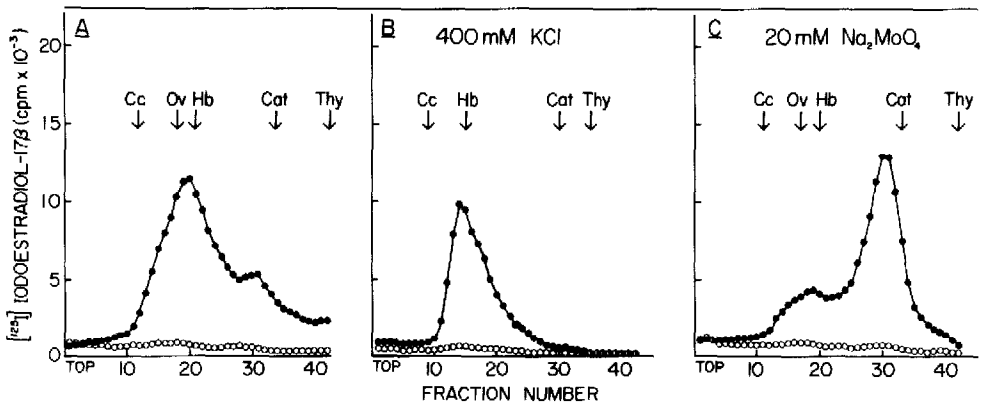

Fig. 5. Influence of potassium chloride and molybdate on the sedimentation properties of estrogen receptors in human breast carcinoma by sucrose density gradient centrifugation. Cytosol preparation and reaction conditions are described in Materials and methods. Human hemoglobin was added to each gradient as an internal standard. The marker proteins cytochrome *c* (Cc), ovalbumin (Ov), hemoglobin (Hb), catalase (Cat), and thyroglobulin (Thy) were separated in an additional gradient. Reactions were performed using 4 n*M* [^{125}I]-iodoestradiol-17β in the presence (○) and absence (●) of a 200-fold excess of diethylsilbestrol. (A) The reaction medium consisted of TEGM buffer whereas centrifugation was performed in sucrose (10—35%) gradients made with TE buffer. A receptor preparation containing 0.8 mg of protein was applied. (B) The initial reaction medium was TEGM buffer but sufficient potassium chloride was added after 1 h incubation to bring the final potassium chloride concentration to 400 m*M*; centrifugation was performed in sucrose (10—35%) gradients made with TEK buffer. Cytosol containing 0.5 mg of protein was applied. (C) The reaction medium consisted of TEGM buffer whereas centrifugation was performed in sucrose (10—35%, w/w) gradients made with TE buffer. The preparation applied to the gradient contained 0.7 mg of protein.

of marker proteins employed in this study. The elution positions of these proteins are indicated in Fig. 6. The sedimentation positions of the same markers on sucrose gradients are indicated in Fig. 5. A comparison of the high-molecular-weight (size) species in Fig. 5A (fraction 31) and Fig. 6A (approximately 18 min) relative to the position of the catalase marker demonstrates the difference observed with these methods. Using HPSEC, the high-molecular-weight species appeared to elute with a Stokes radius of > 61 Å whereas on sucrose gradients the largest isoform sedimented more slowly than catalase (51 Å). This seeming discrepancy is usually resolved by the assumption that the molecule is highly asymmetric and in the shape of a prolate ellipsoid of relatively high frictional ratio (1.65) [21].

The concentration of estrogen receptors determined from profiles generated by the respective separation systems as demonstrated in Figs. 5A and 6A were calculated. The sucrose gradient procedure gave 122 fmol/mg cytosol protein whereas HPSEC gave a level of estrogen receptor equivalent to 99 fmol/mg cytosol protein. The sucrose gradient method revealed that 65% of total specific binding was represented by the 4—5 S species and 20% was represented by the 8—9 S species. On HPSEC, a molecular species indistinguishable from the 4 S species seen on sucrose gradients (data not shown) and the high-molecular-weight species constituted 66% and 18% of the total specific binding, respectively.

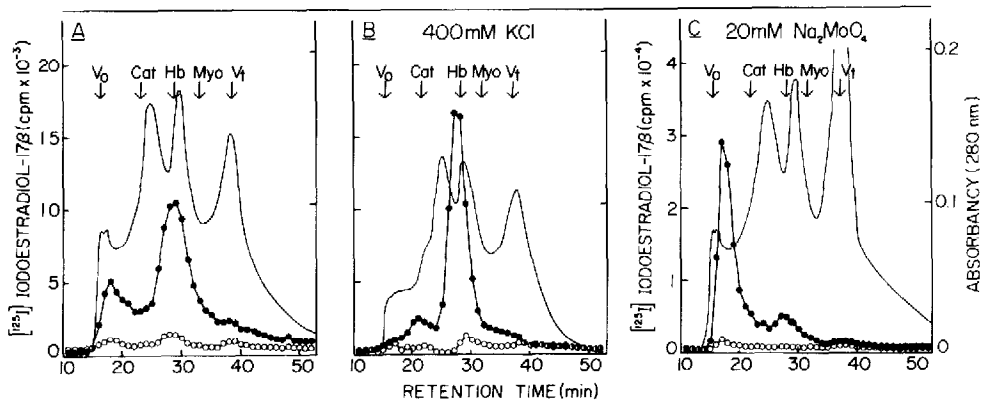

Fig. 6. Influence of potassium chloride and molybdate on the size exclusion properties of estrogen receptors in human breast carcinoma by HPSEC. Cytosol preparation and reaction conditions are described in Materials and methods. In each case, the reactions were performed using 4 n*M* [16α-^{125}I]iodoestradiol-17β as ligand in the presence (○) or absence (●) of a 200-fold excess of diethylstilbestrol. A 100-μl aliquot of each reaction was applied to the column which was eluted at a flow-rate of 0.7 ml/min using $TEGK_{100}$ or $TEGK_{400}$. The marker proteins were chromatographed individually and as a group in separate runs and their retention times were determined from their absorption peaks at 280 nm. The cytosols and reactions were identical to those described in Fig. 5. (A) The reaction medium consisted of TEGM buffer whereas the chromatographic elution buffer was $TEGK_{100}$. A total of 0.8 mg of cytosol protein was applied. (B) The initial reaction medium was TEGM buffer, but sufficient potassium chloride was added after 1 h of incubation to bring the final potassium chloride concentration to 400 n*M*. The chromatographic elution buffer was $TEGK_{400}$ and the quantity of cytosol protein applied was 0.5 mg. (C) The reaction medium consisted of TEGMM buffer whereas the chromatographic elution buffer was $TEGK_{100}$. A total of 0.7 mg of cytosol protein was applied. The trace of eluted materials absorbing at 280 nm is given. Identification of peaks as in Fig. 5. V_0 = void volume as determined using blue dextran; V_t = total volume as determined using ^3H_2O.

Treatment of the cytosol with 400 m*M* potassium chloride resulted in the appearance of a 29—32 Å form on HPSEC which dominated the profile (Fig. 6B). There was a small quantity of a component retained at 22 min which appeared to have a Stokes radius of ~ 48 Å. The sucrose gradient method gave (Fig. 5B) a level of 120 fmol/mg cytosol protein while HPSEC estimated a level of 121 fmol/mg cytosol protein. The gradient technique showed that 88% of the specific estrogen receptor binding was in the 4—5 S form and only 5% was in the 8—9 S form, whereas HPSEC exhibited 85% in the 29—32 Å form and 12% in the high-molecular-weight region.

When 20 m*M* sodium molybdate was added to the homogenizing buffer (TEGM), virtually all of the specific binding was exhibited by a component eluting after the void volume in the HPSEC experiments (Fig. 6C). These data suggest a component with a Stokes radius of > 61 Å. Only a small quantity of the 29—32 Å species remained. A specific estrogen receptor concentration of 150 fmol/mg cytosol protein was estimated by centrifugation whereas HPSEC gave 187 fmol/mg cytosol protein. The higher recovery of receptor by HPSEC relative to that from sucrose gradients may be attributed to the longer incubation time with the stabilizing reagent, sodium molybdate.

The higher level of binding attained in the presence of molybdate was consistent with the stabilizing effects that ion has shown on other receptors [17—20,

22]. Using the sucrose gradient centrifugation, 57% of the specific binding was attributable to the 8—9 S species and 35% to the 4—5 S species, whereas, utilizing HPSEC, 74% of the specific binding corresponds to the high-molecular-weight species and 23% to the 28—32 Å moiety.

Whereas the highly comparable data given in Figs. 5 and 6 are representative of many samples, we have observed several cases where gradients and HPSEC exhibited different profiles. Fig. 7 is a sample of another human breast carcinoma treated in the same manner as that of the sample shown in Fig. 5, i.e. sedimented in the presence of low salt (panel A), adjusted to 400 m*M* potassium chloride and sedimented in high salt (Fig. 5B), or homogenized in molybdate-containing buffers and sedimented on low salt gradients (Fig. 5C). The effects of these treatments are given in Fig. 7 and are qualitatively similar to those illustrated in Fig. 5. The corresponding HPSEC profiles given in Fig. 8 are, however, not nearly as comparable. In general, the profile in Fig. 8A exhibited much less of the 29—32 Å species than would be expected if this species corresponded to the 4—5 S moiety. Fig. 8B demonstrates relatively more of the 48 Å species separating at 22 min than was apparent in the sample shown in Fig. 6B. The high-molecular-weight species (> 61 Å) seen in Fig. 8C appears proportional to that observed on the gradient.

The results of these experiments indicated a common occurrence, namely the tendency for this chromatographic system either to preserve the high-molecular-weight form or precursor, or to induce the aggregation of the

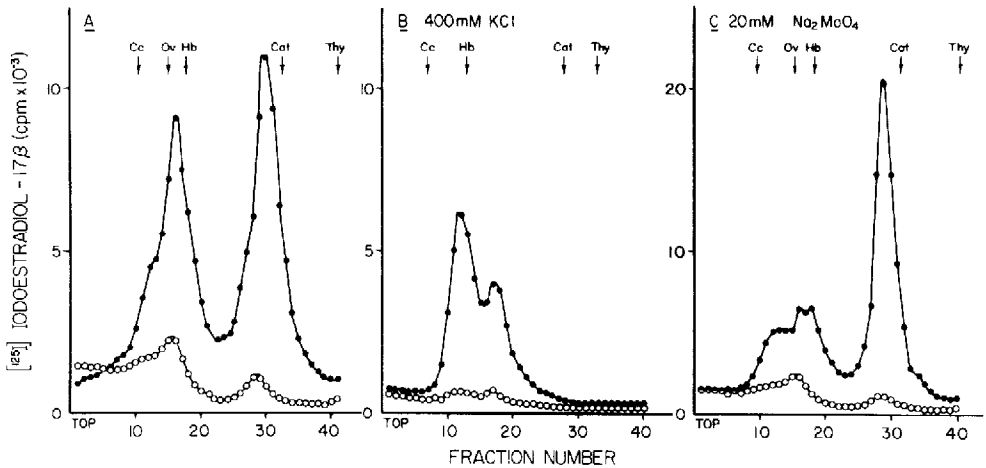

Fig. 7. Influence of potassium chloride and molybdate on the sedimentation properties of estrogen receptor in human breast carcinoma by sucrose density gradient centrifugation. Conditions for cytosol preparation and receptor separation were identical to those given in the legend of Fig. 5. (A) The reaction medium consisted of TEGM buffer whereas centrifugation was performed in sucrose (10—35%) gradients made with TE buffer. A receptor preparation containing 0.8 mg of protein was applied. (B) The initial reaction medium was TEGM buffer but sufficient potassium chloride was added after 1 h incubation to bring the final potassium chloride concentration to 400 m*M*; centrifugation was performed in sucrose (10—35%) gradients made with TEK buffer. Cytosol containing 0.5 mg of protein was applied. (C) The reaction medium consisted of TEGMM buffer whereas centrifugation was performed in sucrose (10—35%, w/w) gradients made with TE buffer. The preparation applied to the column contained 0.8 mg of protein. Identification of peaks as in Fig. 5.

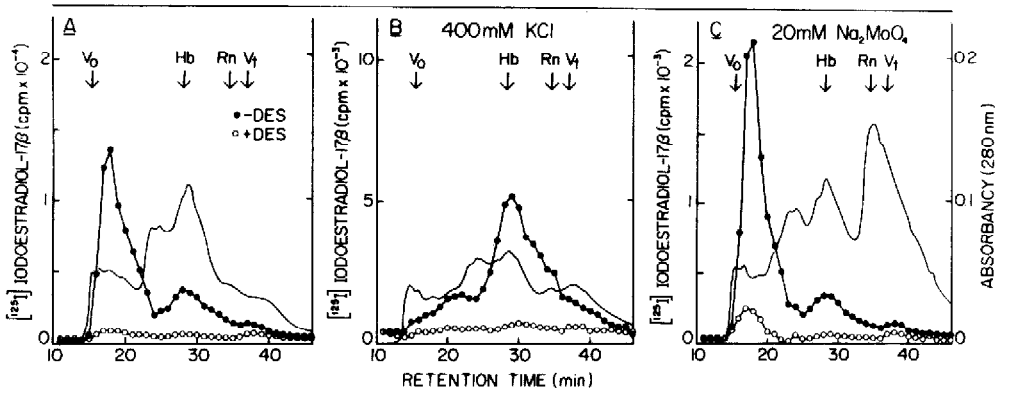

Fig. 8. Influence of potassium chloride and molybdate on the size exclusion properties of estrogen receptors in human breast carcinoma by high-performance liquid chromatography. Conditions for cytosol preparation and receptor separation were identical to those given in the legend of Fig. 6. (A) The reaction medium consisted of TEGM buffer whereas the chromatographic elution buffer was $TEGK_{100}$. A total of 0.8 mg of cytosol protein was applied. (B) The initial reaction medium was TEGM buffer, but sufficient protassium chloride was added after 1 h of incubation to bring the final potassium chloride concentration to 400 n*M*. The chromatographic elution buffer was $TEGK_{400}$ and the quantity of cytosol protein applied was 0.5 mg. (C) The reaction medium consisted of TEGMM buffer whereas the chromatographic elution buffer was $TEGK_{100}$. In this case 0.8 mg of cytosol protein was applied. The trace of eluted materials absorbing at 280 nm is given. V_0 = void volume as determined using blue dextran; V_t = total volume as determined using ^3H_2O; Hb = hemoglobin; Rn = ribonuclease; DES = diethylstilbestrol.

receptor. Since molybdate is considered to be a potential inhibitor of protease activity as well as of phosphatase action [22, 23], our finding of a high-molecular-weight species supports the concept of a processing step in the steroid hormone receptor cascade. In summary, HPSEC separates the various species of receptors for the sex-steroid hormones on the basis of size and shape. Furthermore, the speed and efficiency of HPSEC in combination with [^{125}I]-iodoestradiol-17β of high-specific radioactivity should permit sequential analyses of estrogen receptor isoforms prepared by a variety of methods.

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