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ISOLATION OF UNLIGANDED STEROID RECEPTOR PROTEINS BY HIGH-PERFORMANCE SIZE-EXCLUSION CHROMATOGRAPHY

AN INVESTIGATION OF STEROID-DEPENDENT STRUCTURAL ALTERATIONS

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SUMMARY

The physicochemical properties of unliganded steroid receptor proteins remain largely unknown primarily due to receptor lability in the absence of specific ligand, especially during prolonged biochemical analyses. We have utilized high-performance size-exclusion chromatography (HPSEC) as a rapid means of investigating the structural properties of cytosolic estrogen receptor proteins in both the presence and absence of [^{3}H]estradiol-17β. Cytosols prepared from immature calf uteri were analyzed by HPSEC on an Altex TSK-3000 SW column (600 mm × 7.5 mm I.D.) either before or after incubation with 10 n*M* [^{3}H]estradiol-17β. Postcolumn detection of previously unliganded receptor was accomplished by incubation of fractions (0.38 ml) with 10 n*M* [^{3}H]estradiol-17β for 2–18 h at 0°C. Receptor-bound steroid was separated from free steroid by incubation with small pellets of hydroxylapatite. Nonspecific binding of [^{3}H]estradiol-17β in parallel fractions was estimated using an unlabelled competitor (diethylstilbestrol) specific for the estrogen receptor. In low ionic strengths the receptor exists as a single, relatively stable, large form (retention time 34 min). The elution properties of this receptor configuration do not depend on bound steroid ligand. Analysis of receptor at elevated ionic strengths in the presence and absence of steroid ligand suggests that the salt-induced dissociation of receptor components to smaller forms (retention time 47 min) may be partially steroid-dependent. Characterization of receptor in 6 *M* urea demonstrates the presence of intermediate-sized receptor components (retention time 36–38 min). Analyses of receptor in 6 *M* urea–0.4 *M* potassium chloride suggests an inhibition of the more extensive salt-induced dissociation event seen in 0.4 *M* potassium chloride alone. Furthermore, the intermediate-sized receptor forms seen under these conditions (retention time 41–42 min) are generated in a steroid-dependent manner. The preparation of different molecular forms of biologically active, unliganded estrogen recep-

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tor by HPSEC should help further our investigations into the molecular mechanism(s) by which steroid hormones exert their receptor-mediated effects on target cells.

INTRODUCTION

Elucidation of the structure of steroid receptor proteins remains an area of intense investigation. Receptors are a dynamic set of steroid-specific proteins exhibiting relative extremes not only in the lability of their steroid-binding properties, but also in their sensitivity to extensive size and surface charge modifications both *in vivo* and *in vitro*[1–4]. Most efforts to identify the undegraded state of a given receptor have been based upon estimations of size, relying heavily upon the inclusion of various receptor-stabilizing agents[2,3]. These findings have tempted investigators to equate the large, undegraded receptor form with the native form. However, the native or untransformed state of steroid–receptor complexes has been defined almost exclusively, not by size, but by surface ionic properties (relatively acidic) and low affinity for nuclei, DNA or ATP[3,4]. Transformation of steroid–receptor complexes is basically defined as an acquired high affinity for nuclei, DNA or other polyanionic structures[3,4]. This is a steroid-dependent event thought to precede steroid–receptor influence on gene expression.

There is now substantial evidence to support the general application of models depicting the steroid-dependent receptor transformation event as one accompanied by a significant decrease in apparent molecular weight[3,5–12]. If this decrease in molecular weight is an integral part of the steroid-dependent transformation mechanism, then receptor-stabilizing agents which maintain receptors in a large (undissociated) configuration may also prevent transformation. This is known to be the case for the commonly used receptor-stabilizing agent, sodium molybdate[2–5,12–17]. However, any structural similarities between the molybdate-stabilized, estrogen-receptor complex and the native, unliganded receptor remain unknown.

We have proposed[6,7,9] that receptor transformation may be a reversible process involving the non-enzymatic association–dissociation of heterologous steroid-binding and non-steroid-binding components (subunits). It is important in our studies to distinguish receptor transformation events from nonspecific degradation events. Therefore, we are examining steroid-dependent alterations which are reversible *in vitro*. To further our investigation of steroid-dependent structural alterations in receptor (including transformation) it is necessary to isolate and characterize these receptor proteins in the unliganded, native (untransformed) state. The physicochemical properties of unliganded receptor proteins are virtually unknown since most physical investigations have utilized liganded steroid–receptor complexes. We report here our preliminary characterization of unliganded calf uterine estrogen receptor by high-performance size-exclusion chromatography (HPSEC) under conditions which appear to perturb receptor structure in a steroid-dependent manner.

EXPERIMENTAL

Materials

Uteri from small, immature calves were obtained from local slaughterhouses. The

uterine horns (7–15 g) were rinsed immediately after removal in ice-cold saline, cut into 1-g pieces, frozen in liquid nitrogen (or dry ice) and stored frozen at −85°C. Altex TSK-3000 SW high-performance size-exclusion columns (600 mm × 7.6 mm I.D.) were purchased from Beckman. [^{3}H]Estradiol-17β (90–100 Ci/mmole) was purchased from New England Nuclear. Trizma base, diethylstilbestrol (DES), dithiothreitol (DTT) and glycerol were from Sigma. Disodium ethylenediaminetetraacetic acid (Na_2EDTA) was from Fisher Scientific. Urea was purchased from Bethesda Research Labs. Prepacked Sephadex G-25 (PD-10) columns (6 cm × 1.5 cm I.D.) were obtained from Pharmacia.

Preparation of cytosol and labelling of estrogen binding proteins

All procedures were performed in a cold room at 0–6°C. Pieces of frozen uteri were minced before being homogenized in 2 volumes of 10 m*M* Tris–HCl buffer (pH 7.4–7.8 at 0°C) containing 1 m*M* DTT and 20% glycerol (by volume). Cytosol (10–15 mg protein/ml) was obtained by high speed centrifugation (100 000 *g*; 60 min) of the homogenate and labelled at 0°C with 5–10 n*M* [^{3}H]estradiol-17β in the presence (nonspecific binding) or absence (total binding) of a 100-fold molar excess of the radioinert competitor DES. Where noted, immediately before HPSEC, radiolabelled steroid–protein complexes were quickly (< 5 min) separated from excess free steroid by rapid chromatography on small columns (9 ml) of G-25 Sephadex (Pharmacia PD-10 columns).

High-performance size-exclusion chromatography

HPSEC was performed using a Beckman Model 110A high-performance liquid chromatography (HPLC) pump (in a cold room at 0–6°C) and an Altex TSK-3000 SW column (600 mm × 7.6 mm I.D.) equipped with in-line frit filters and a guard column (100 mm × 7.6 mm I.D.). Cytosolic estrogen binding proteins prelabelled with [^{3}H]estradiol-17β were cleared of excess free [^{3}H]estradiol-17β by rapid chromatography on small (9 ml) Sephadex G-25 columns (PD-10). Unliganded estrogen receptor preparations were treated similarly. Samples (500 μl) were injected with Rheodyne 7125-S injectors and 1.0-min fractions were collected at 0.380 ml/min. The column (TSK-3000 SW) was equilibrated with 50 m*M* potassium phosphate buffer (pH 7.4 at 0°C) containing 1.5 m*M* Na_2EDTA, 1 m*M* DTT and 10% glycerol (v/v) and calibrated with 6–9 purified proteins of known Stokes radii ranging from 18Å to 86Å (see Fig. 9). Where noted, the HPSEC elution buffer additionally contained 10 m*M* sodium molybdate, 0.4 *M* potassium chloride or 6 *M* urea (with and without 0.4 *M* potassium chloride). Column void volumes (V_0) were determined by exclusion of Blue-Dextran 2000 or thyroglobulin. The retention time of molecules eluting in the inclusion volume was estimated using β-mercaptoethanol and [^{3}H]estradiol-17β to be 73 min. The column was frequently cleaned with 1 *M* potassium chloride and stored in methanol when not in use. The column calibration was checked periodically and plots of log Stokes radii *versus* retention time (or elution volume) were linear with correlation coefficients (r values) typically better than 0.95.

Postcolumn detection (postlabelling) of estrogen receptor proteins: hydroxylapatite batch adsorption assay

All portions of this assay were conducted at 0°C. Fractions (1 ml) of estrogen

receptor preparations (cytosol) analyzed by HPSEC in the unliganded (unlabelled) form were collected into 100 μl of buffer [50 m*M* potassium phosphate (pH 7.4), 1.5 m*M* Na_2EDTA, 1 m*M* DTT and 20% glycerol] containing 10 n*M* [^{3}H]estradiol-17β. To determine nonspecific binding of [^{3}H]estradiol-17β, alternate fractions additionally contained a 100-fold molar excess of the estrogen receptor-specific competitor, DES. The collected fractions (now 0.480 ml) were incubated for 2–24 h at 0°C before 500 μl of a 50% slurry of hydroxylapatite (Bio-Rad) were added to each fraction. The fractions were incubated with hydroxylapatite for 20–30 min before the addition of 2 ml of ice-cold distilled water. Incubation was continued for another 20–30 min. The hydroxylapatite was pelleted by centrifugation and resuspended in 4 ml of wash buffer containing 10 m*M* Tris–HCl (pH 7.4 at 0°C), 1 m*M* DTT, 20% glycerol (by volume) and 1% Tween-80. This wash procedure was performed 4 times (16 ml wash volume) or until the supernate contained negligible radioactivity. To extract bound radioactivity, the final pellet of hydroxylapatite was resuspended into 1 ml of ethanol at 25°C and incubated for 30–45 min (25°C) with mixing every 5–10 min. The hydroxylapatite was pelleted by centrifugation and the ethanol supernate (1 ml) was decanted into scintillation vials. Atomlight scintillation cocktail (New England Nuclear) was added (3 ml) for liquid scintillation spectrophotometry. A Beckman Model LS-250 liquid scintillation system was used with a counting efficiency that averaged 25–26% for postlabelled HPSEC fractions (due to ethanol) compared to 35–37% for prelabelled HPSEC fractions. Profiles shown are uncorrected for quench.

RESULTS

Calf uterine estrogen receptors have been analyzed and isolated preparatively by HPSEC in both the liganded and unliganded forms. Under a variety of experimental conditions shown to perturb receptor structure (size), yet maintain biological activity, we have observed what appear to be both steroid-independent and steroid-dependent structural alterations.

As shown in Fig. 1, cytosolic calf uterine estrogen receptor elutes as a single large form (retention time 34 min). The elution properties and recovery (80–100%) of this receptor form appear unchanged from 2 to 24 h of incubation at 0°C with steroid. The reproducible (n = 7) elution of this large form of calf uterine estrogen receptor has been independently verified by size-exclusion chromatography on Sephacryl S-300 columns (data not shown). There is little evidence for the presence of significant quantities of smaller steroid binding proteins or fragments. Nonspecific binding of [^{3}H]estradiol-17β appears minimal or absent when calf uterine estrogen receptor is analyzed by HPSEC under these conditions. The profiles shown in Fig. 2 demonstrate the experimentally manipulated, extreme forms of receptor size not generally attributed to irreversible changes in receptor structure. The use of a commonly employed receptor-stabilizing agent, sodium molybdate (10 m*M*), did not influence the HPSEC elution profile of calf uterine estrogen receptor. However, inclusion of 0.4 *M* potassium chloride in the HPSEC elution buffer dissociates the major portion of receptor to a smaller form(s) eluting at 47 min. There appears to be a salt-stable, intermediate-sized receptor form eluting at 38–40 min as well as low quantities of residual large receptor form eluting at 34 min. The intermediate-sized and salt-stable receptor forms are present whether the cytosol is injected directly into the

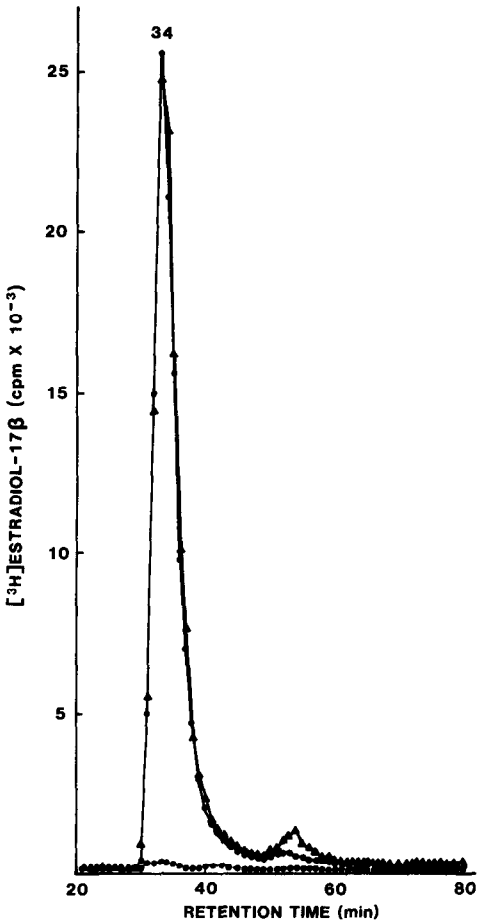

Fig. 1. HPSEC of calf uterine estrogen receptor: stability of large receptor form with time. Cytosol was prepared and labelled with 10 n*M* [³H]estradiol-17*β* for 2 h (●—●) or 24 h (▲—▲) at 0°C. Nonspecific [³H]estradiol-17*β* binding activity (○---○) was determined in parallel incubations using an unlabelled competitor (DES) specific for estrogen receptor (24 h only). Protein-bound [³H]estradiol-17*β* was separated from free [³H]estradiol-17*β* by Sephadex G-25 chromatography immediately prior to HPSEC. Recovery was calculated to be 104% (2 h) and 81% (24 h). Details of HPSEC are provided in Experimental. The retention time of estrogen receptor is shown (min).

column with no previous exposure to 0.4 *M* potassium chloride (see Fig. 5 left) or previously equilibrated with 0.4 *M* potassium chloride elution buffer as described in the legend to Fig. 2.

The basic HPSEC elution profile of calf uterine estrogen receptor appears not to depend on bound steroid ligand. As illustrated in Fig. 3, the elution behavior of receptor prelabelled with [³H]estradiol-17*β* is nearly indistinguishable from the elution behavior of unliganded (unlabelled) receptor. It is noteworthy that postlabelling fractions of receptor preparations analyzed by HPSEC in the absence of added steroid failed to reveal additional nonspecific binding components or smaller receptor

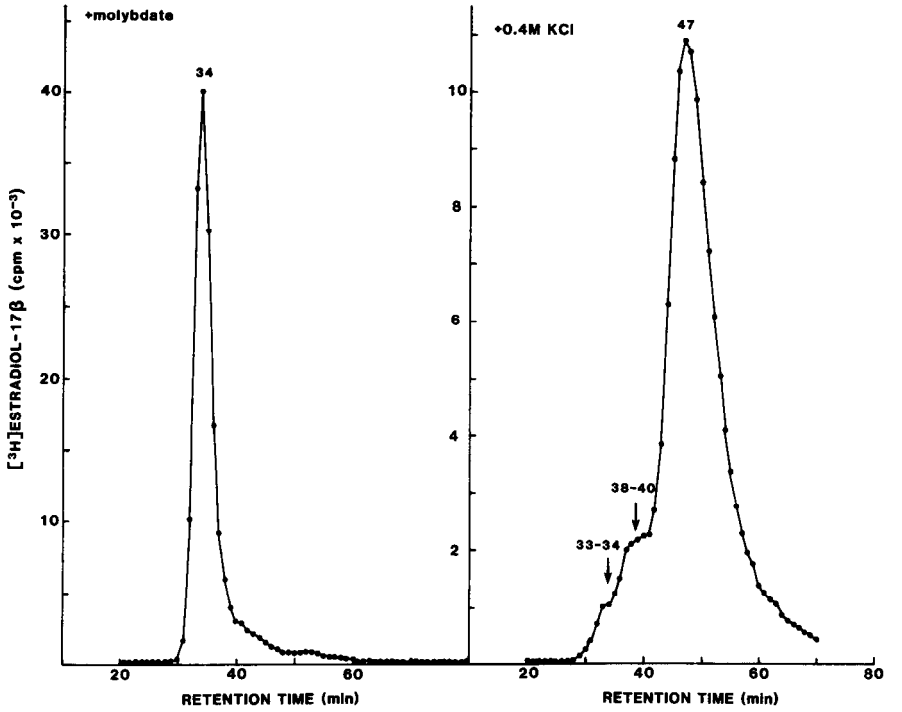

Fig. 2. HPSEC of calf uterine estrogen receptor: analysis of molybdate-stabilized form (left) and 0.4 *M* potassium chloride-dissociated forms (right). Cytosol was prepared both with (left) and without (right) 10 m*M* sodium molybdate in the homogenizing buffer and incubated with 10 n*M* [^{3}H]estradiol-17β at 0°C. Immediately prior to HPSEC, the labelled receptor preparations were subjected to chromatography on small (10 ml) columns of Sephadex G-25 equilibrated with HPSEC elution buffer containing 10 m*M* sodium molybdate (left) or 0.4 *M* potassium chloride (right). Subsequent HPSEC analyses were in these same buffers. Details of HPSEC are provided in Experimental. The retention times (min) of estrogen receptor forms are shown.

forms (fragments). However, parallel analyses of liganded and unliganded receptor preparations under these same conditions may reveal steroid-dependent alterations in receptor size. As shown in Fig. 4, even though the prelabelled estrogen–receptor complex elutes as a single component at 34 min there appear to be variations in receptor structure which only become apparent during analysis of receptor in the unliganded form. A comparison of these results (n = 1) with the more typical (n = 11) results as presented in Fig. 3 suggests possible variations in receptor stability from different calf uterine tissues. Analyses of receptor by high-performance chromatofocusing demonstrate that receptor surface charge (or stability) may vary in a tissue dependent manner despite efforts to collect calf uterine tissues from closely matched animals[13].

Separation of salt-dissociated receptor forms in the presence (prelabelled) and absence (postlabelled) of [^{3}H]estradiol-17β provides evidence that salt-induced dissociation of receptor components may be partially steroid-dependent. As shown in Fig. 5, the relative ratio of undissociated (34 min) to fully dissociated (47 min) forms

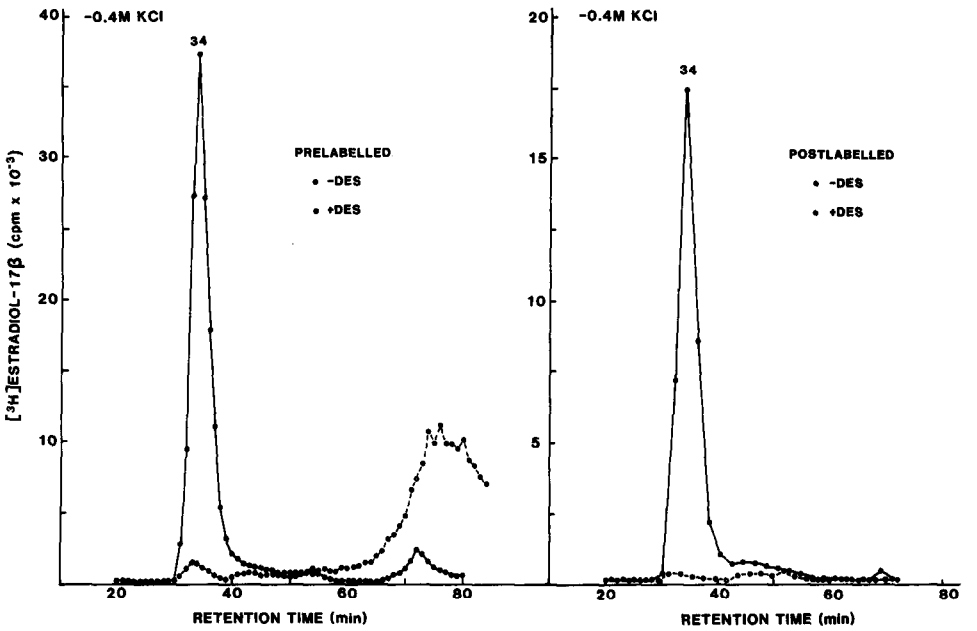

Fig. 3. HPSEC of calf uterine estrogen receptor in the presence (prelabelled) and absence (postlabelled) of [^{3}H]estradiol-17β. Cytosol was prepared and prelabelled with 10 nM [^{3}H]estradiol-17β (left) or analyzed directly in the unlabelled form (right). Neither receptor preparation was chromatographed on Sephadex G-25 prior to HPSEC. Post-column detection of estrogen receptor eluting in the unlabelled (unliganded) form (right) was by incubation with 5 nM [^{3}H]estradiol-17β in the presence (nonspecific binding, O---O) or absence (total binding, ●—●) of a 100-fold molar excess of radioinert competitor, DES. Separation of receptor-bound from free [^{3}H]estradiol-17β was by a hydroxylapatite batch-adsorption assay as described in Experimental. The quantity of specific steroid-binding activity indicated is approximately 65% of that shown for the prelabelled control experiment (left). Details of HPSEC are as described in Experimental. Column retention times for the major estrogen receptor form are shown. Free [^{3}H]estradiol-17β elutes as a broad peak at approximately 73 min when not removed prior to HPSEC.

appears greater when receptor is analyzed in the absence of steroid. As will be shown, this effect is more evident when 6 M urea is included in the 0.4 M potassium chloride elution buffer. Note that free [^{3}H]estradiol-17β which was not removed prior to HPSEC for these experiments elutes as a broad peak at approximately 73 min. Elution of β-mercaptoethanol at 73 min demonstrates that this is the inclusion volume. Elevated levels of [^{3}H]estradiol-17β in the inclusion volume during HPSEC of samples labelled in the presence of DES indicate free steroid as well as steroid wich may have dissociated from low affinity, nonspecific binding proteins during HPSEC. Estimated quantities of [^{3}H]estradiol-17β associated nonspecifically with low affinity, high capacity, non-receptor binding proteins (see Experimental) are consistently lower or negligible by HPSEC compared to determinations by Sephadex G-25 chromatography or the dextran–charcoal adsorption method (unpublished observation). We have previously demonstrated the significant steroid binding capacity of other silica-based high performance columns[14–15].

As shown in Fig. 6, using 0.4 M potassium chloride, it is possible to isolate

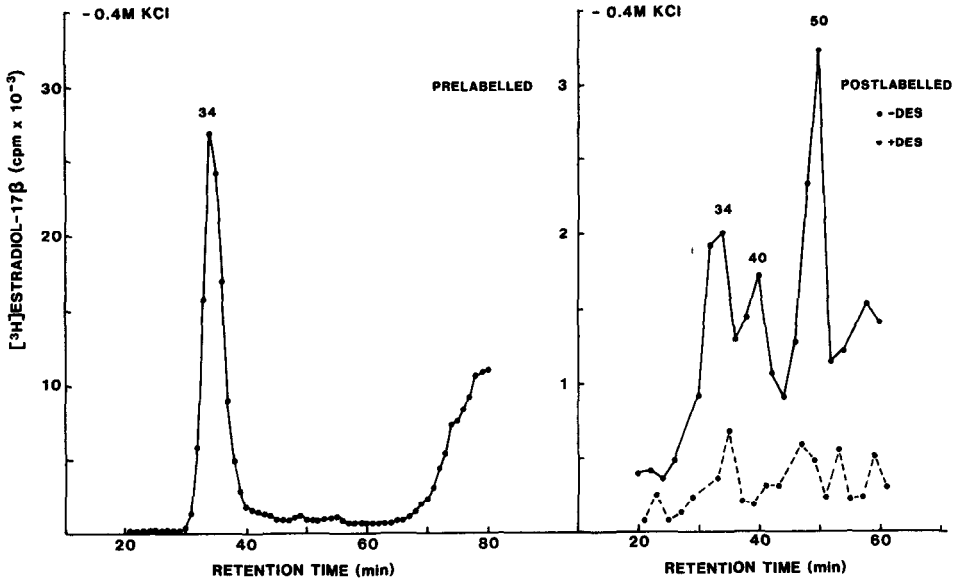

Fig. 4. HPSEC of calf uterine estrogen receptor: postcolumn detection of modified receptor forms. Experimental procedures are exactly as those described in the legend to Fig. 3. Cytosolic estrogen receptor preparations, prelabelled with 10 n*M* [³H]estradiol-17*β*, were analyzed by HPSEC (left) as described in the legend to Fig. 3. Aliquots of the same receptor preparation were analyzed by HPSEC in the unliganded form (right). Postcolumn detection of estrogen receptor in these fractions was as described in Experimental and the legend to Fig. 3. Total (●—●) and nonspecifically (○---○) bound [³H]estradiol-17*β* reveal estrogen receptor forms eluting with various retention times (shown).

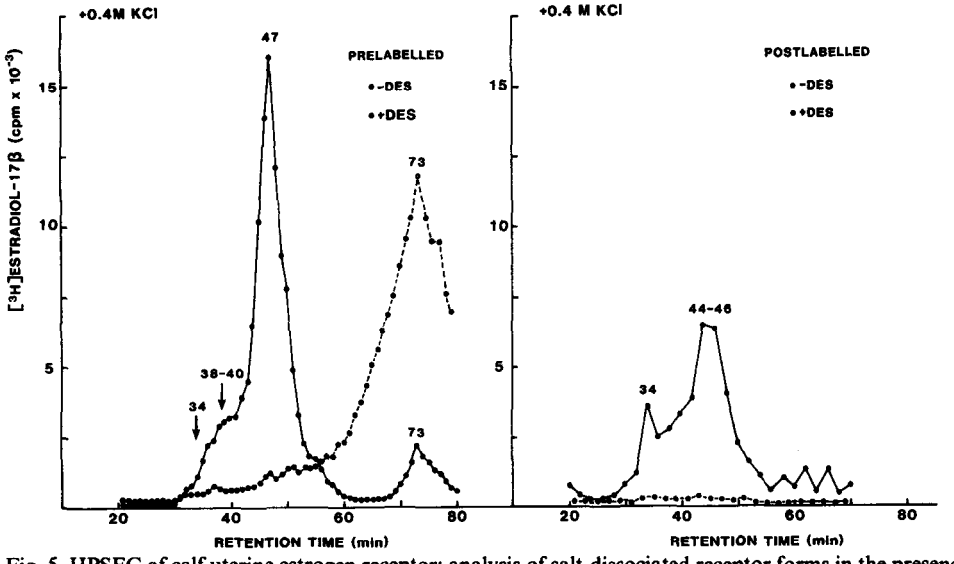

Fig. 5. HPSEC of calf uterine estrogen receptor: analysis of salt-dissociated receptor forms in the presence (prelabelled) and absence (postlabelled) of [³H]estradiol-17*β*. Cytosol was prepared and prelabelled with 10 n*M* [³H]estradiol-17*β* (left) or analyzed in the unlabelled (unliganded) form (right). Details of HPSEC with 0.4 *M* potassium chloride elution buffer are described in Experimental. Postcolumn detection of both total (●—●) and nonspecifically bound (○—○) [³H]estradiol-17*β* was as described in Experimental and the legend to Fig. 3. Retention times of estrogen receptor forms are shown. The retention time of free [³H]estradiol-17*β* is 73 min. Note the partial steroid-dependence of the salt-induced dissociation of large (34 min) to small (47 min) receptor forms.

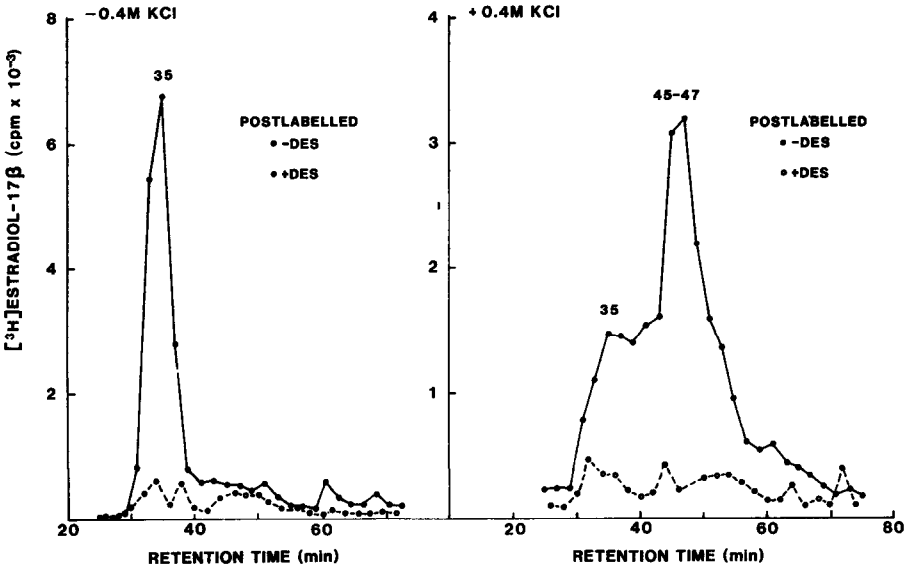

Fig. 6. HPSEC of calf uterine estrogen receptor: preferential isolation of unmodified (large) and salt-dissociated (small) receptor forms in the absence of steroid ligand. Cytosol was prepared and analyzed in the unlabelled form by HPSEC in the absence (left) or presence (right) of 0.4 *M* potassium chloride. Immediately prior to HPSEC, receptor preparations were equilibrated into the appropriate HPSEC elution buffers by rapid chromatography through small (10 ml) columns of Sephadex G-25 previously equilibrated in these same buffers. Details of HPSEC and post-column detection of total (●—●) and nonspecific (○—○) [³H]estradiol-17β-binding proteins are provided in Experimental. Retention times (min) of specific estrogen receptor forms are shown.

both large and small forms of estrogen receptor from a given tissue in the unliganded form with retention of biological activity (steroid-binding). However, as shown in Figs. 7 and 8, there may be a more discriminating means of controlling dissociation of the unliganded receptor form.

HPSEC of prelabelled calf uterine estrogen receptor in the presence of 6 *M* urea reveals a receptor form(s) of intermediate size. Relative to the sharp elution (34 min) profiles shown in Figs. 1 and 3, in 6 *M* urea the peak is broad with distinct shoulders suggesting a heterologous set of components. This is a consistent observation ($n = 4$). HPSEC of unliganded receptor with subsequent detection of receptor by postlabelling yields a slightly different receptor elution profile. These experiments suggest again that receptor sensitivity to *in vitro* physical perturbations may be affected by bound steroid. In separate experiments (not shown) removal of urea by Sephadex G-25 chromatography results in the receptor eluting as a large (or possibly aggregated) form (33 min).

HPSEC of calf uterine estrogen receptor in the presence of both 6 *M* urea and 0.4 *M* potassium chloride demonstrates that 6 *M* urea prevents the salt-dependent dissociation of receptor into the smaller (47 min) component parts seen in Fig. 2. Fig. 8 illustrates the combined urea–salt effects on receptor during HPSEC. A comparison of estrogen receptor preparations analyzed in 6 *M* urea–0.4 *M* potassium chloride in the presence (liganded) and absence (unliganded) of steroid demonstrates

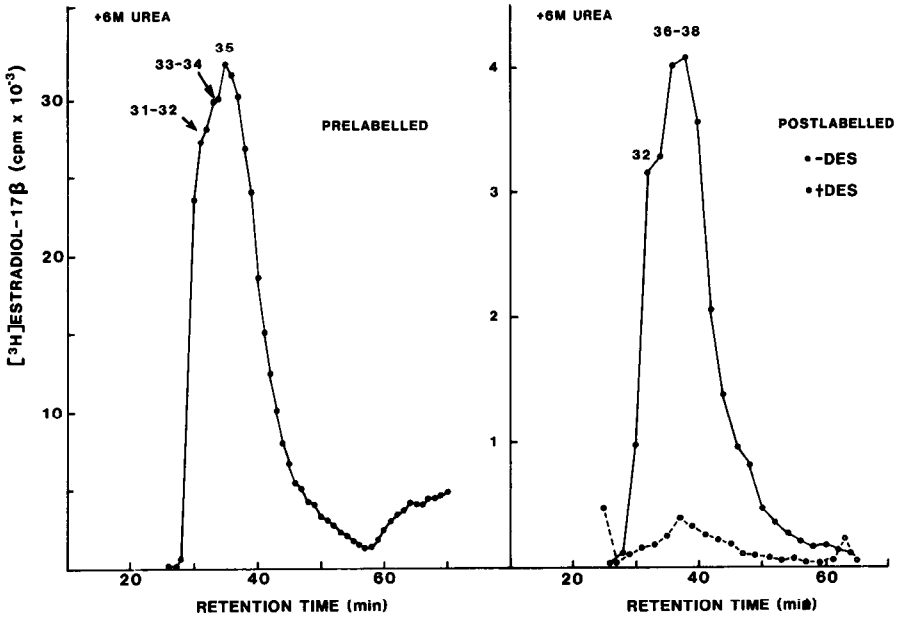

Fig. 7. HPSEC of calf uterine estrogen receptor: partial steroid-dependence of 6 *M* urea-induced dissociation of receptor components. Cytosol was prepared and either prelabelled (left) with 10 n*M* [^{3}H]estradiol-17β (see Experimental) or analyzed in the unlabelled form (right). HPSEC was performed using elution buffer containing 6 *M* urea. Postcolumn detection of estrogen receptor was as described in Experimental and the legend to Fig. 3. Nonspecific [^{3}H]estradiol-17β-binding components (O—O) were identified using a radioinert competitor specific for estrogen receptor, DES. Details of HPSEC are provided in Experimental. The retention times (min) of estrogen receptor forms are shown.

that these structural alterations may be steroid dependent. The elution of prelabelled receptor at 41–42 min indicates a receptor form of intermediate size between that observed in 6 *M* urea alone (36–38 min) and that observed in 0.4 *M* potassium chloride alone (47 min). There are at least three additional receptor forms indicated (*i.e.* 46, 48–49 and 58–59 min) which do not appear to be present when receptor is analyzed under these conditions in the absence of steroid ligand. Interestingly, the profile of unliganded receptor in 6 *M* urea–0.4 *M* potassium chloride is similar to that of unliganded receptor analyzed in 6 *M* urea alone (Fig. 7). The observation of steroid-dependent elution behavior in urea–salt appears reproducible (n = 3). The 6 *M* urea–0.4 *M* potassium chloride effects on receptor size as determined by HPSEC appear to be fully reversible (data not shown).

The influence of 6 *M* urea on the elution behavior of standard proteins with known Stokes radii (18 to 86Å) were minimal and not enough to account for the specific urea effects on the receptor preparations analyzed. The calibration profile for the TSK-3000 SW column is shown for low and high salt elution buffers (Fig. 9). Inclusion of 6 *M* urea in the low salt elution buffer maintains the linear profile but with a gradual shift (decrease) in retention time of up to 2–3 min for cytochrome *c*. In the high salt elution buffer (0.4 *M* potassium chloride), inclusion of 6 *M* urea had a similar effect except that the maximum displacement (decrease) of retention time was 1–1.5 min (data not shown).

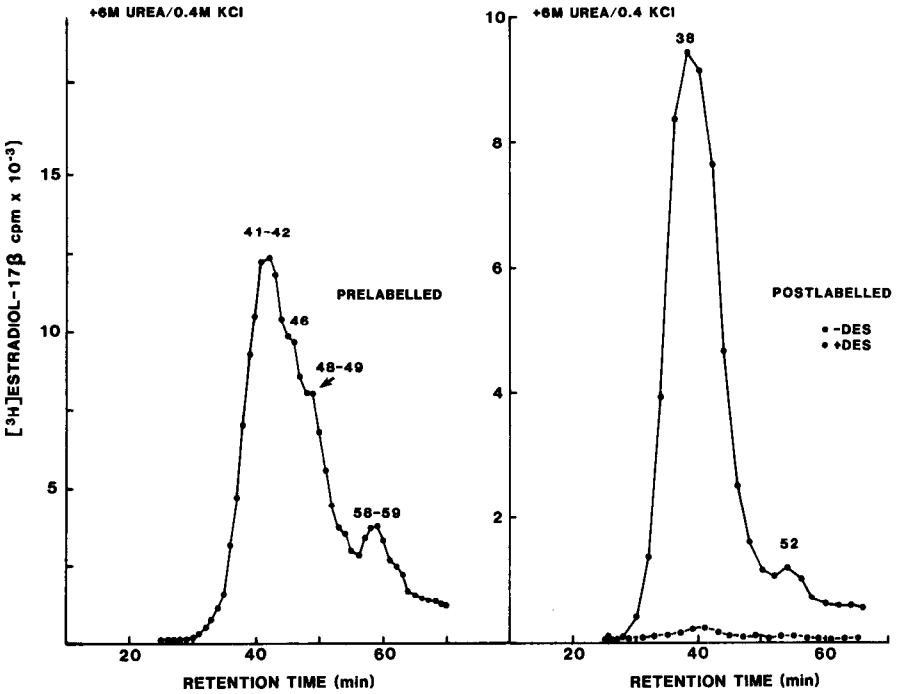

Fig. 8. HPSEC of calf uterine estrogen receptor: steroid-dependence of urea–potassium chloride-induced dissociation of receptor components. Cytosol was prepared and either prelabelled with 10 n*M* [^{3}H]estradiol-17β as described in Experimental (left) or analyzed in the unlabelled form (right) using postcolumn receptor-labelling techniques. Labelled and unlabelled receptor preparations were equilibrated by rapid G-25 chromatography into HPSEC elution buffer containing 6 *M* urea and 0.4 *M* potassium chloride. Postcolumn detection of estrogen receptor was as described in Experimental and the legend to Fig. 3. Nonspecific [^{3}H]estradiol-17β-binding components (O—O) were identified in postlabelled fractions using a radioinert, estrogen-specific competitor, DES. Details of HPSEC are provided in Experimental. Retention times (min) of estrogen receptors are shown.

DISCUSSION AND CONCLUSIONS

We have used HPSEC as a rapid analytical and preparative means of examining the structure of estrogen receptor proteins in cytosol prepared from immature calf uteri. For the first time we are now able to characterize certain physical properties of the estrogen receptor in both the presence and absence of its specific steroid ligand, [^{3}H]estradiol-17β. We believe that physical characterization of unliganded (native) receptor is necessary if we are to understand steroid-dependent alterations in receptor structure and function (*i.e.* transformation).

In the presence of ligand, the receptor appears in a relatively stable, large configuration. When receptor preparations are prepared and analyzed by HPSEC in the presence of 10 m*M* sodium molybdate, there is no change in the receptor elution profile. Since molybdate has been effectively used to prevent receptor "aggregation" as well as to stabilize the receptor against dissociation and/or degradation events[1–3] we believe our HPSEC results suggest that the calf uterine estrogen receptor is par-

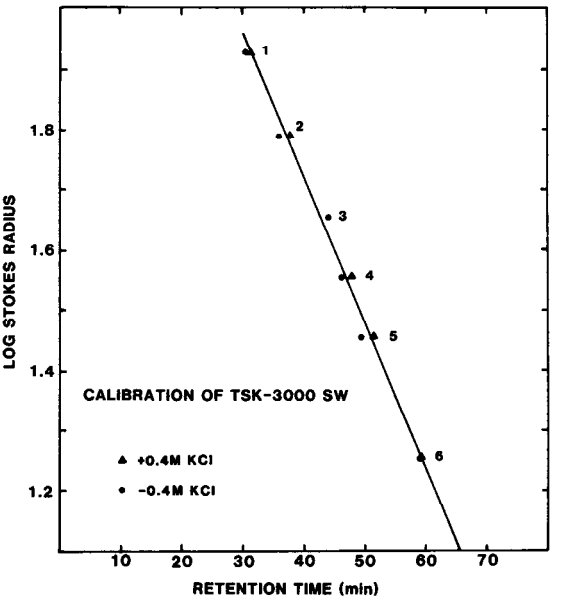

Fig. 9. Calibration of TSK-3000 SW high-performance size-exclusion column with proteins of known physical properties. (1) Void volume [thyroglobulin (85 Å) or Blue Dextran 2000]; (2) ferritin (61.5 Å); (3) aldolase (45 Å); (4) bovine serum albumin (35.9 Å); (5) ovalbumin (28.6 Å); (6) cytochrome *c* (17.9 Å). The retention time of smaller molecules in the inclusion volumes were estimated by elution of β-mercaptoethanol and [^{3}H]estradiol-17β to be 73 min. Details are described in Experimental.

ticularly stable under the experimental conditions we have utilized. However, the specific structure and possible subunit organization of this large form of receptor is unknown. Also unknown is the possible influence of specific ligand (estradiol) on this receptor structure and any subsequent processing which may occur during its mediation of steroid hormone effects.

We have shown by HPSEC that the receptor elution profile is typically unchanged when receptor is analyzed at low ionic strengths in the unliganded form. Therefore, we conclude that the large form (34 min) of receptor is stable as such in the presence or absence of specific ligand. This type of rapid purification procedure may be an efficient means of preparing unliganded receptor preparations for more specific characterization with affinity-labelling receptor agonists such as tamoxifen aziridine[16]. HPSEC of unliganded (or unlabelled) receptor and the use of postcolumn-detection (postlabelling) techniques additionally helps to confirm the absence of lower affinity, smaller receptor forms (or steroid-binding fragments) as well as "nonspecific" steroid-binding proteins. Given the demonstrated capacity of silica-based HPLC columns (*i.e.* TSK-3000 SW) to bind [^{3}H]estradiol-17β[14,15], it may be argued that analyses of prelabelled receptor preparations may not reveal the lower affinity binding components because of steroid dissociation during HPSEC. Postlabelling fractions of unliganded receptor obtained by HPSEC has indeed revealed tissue related variations in the size distribution (stability) of receptor which were unapparent when prelabelled receptor preparations were similarly analyzed.

In the presence of ligand, the receptor is quickly (*ca.* 34 min) and rather efficiently dissociated into a smaller form upon exposure to elevated ionic strength (0.4 *M* potassium chloride). The shift in retention time (34 to 47 min) is reproducible and occurs whether or not the receptor has been equilibrated with buffer containing 0.4 *M* potassium chloride prior to HPSEC. The presence of an intermediate-sized receptor form (38–40 min) in 0.4 *M* potassium chloride suggests that the large form of receptor (34 min) may be composed of more than one steroid-binding subunit. However, it is also possible that the smaller receptor form (47 min) is associated (in an as yet unknown stoichiometry) with other non-steroid-binding proteins to form the large receptor species observed in lower ionic strength buffers. Analyses of unliganded receptor by HPSEC in 0.4 *M* potassium chloride containing buffer suggests that the salt-induced dissociation of large receptor (34 min) to a smaller form(s) may be influenced by the presence of specifically bound steroid.

Salt-induced dissociation of the larger receptor form appears to be more complete in the presence than in the absence of specifically bound ligand. These results are interesting relative to the receptor transformation concept. The steroid-dependence of receptor transformation *in vivo*[17,18] and *in vitro*[3,4] remains relatively undisputed. The net decrease in negative charge associated with transformation is well documented[3,4,17,18]. We have previously noted the correlation between this change in surface charge and the dramatic decrease in molecular weight which appears to occur coincidently during transformation[5–9,10–12]. Based on our results with the salt-induced transformation of glucocorticoid receptor[9] and given the near universal decrease in receptor size accompanying transformation[10–12], we expected the "steroid-dependence" of the salt-induced decrease in receptor size (Fig. 6) to be more pronounced. It may be, however, that the general stability of receptor proteins in their large, untransformed (native) state varies with individual receptor species and their target tissues, especially in unpurified cytosol preparations. There is clear precedent for this[2,3,19]. We are also investigating the possibility that endogenous ligand may be responsible in part for the salt-induced dissociation of receptor observed here in the absence of exogenous [^{3}H]estradiol-17β. However, we have labelled our fractionated receptor preparations under non-exchange conditions at 0°C. It is also difficult at this time to exclude the possibility that the large (34 min) and salt-dissociated (47 min) forms of receptor are differentially stable in the unliganded form at elevated ionic strengths. Our ability to preparatively separate and quantify unliganded receptor in both the large and salt-dissociated, smaller form should allow this question to be addressed in future experiments.

We feel that analyses of receptor by HPSEC in 6 *M* urea have resulted in a series of revealing observations. We are not aware of any other attempts to examine receptor structure at urea concentrations above 1–3 *M*(ref. 20). We have demonstrated that both the liganded and unliganded forms of receptor retain their ability to specifically bind [^{3}H]estradiol-17β even after prolonged exposure to 6 *M* urea (at 0°C). This would suggest that the receptor's steroid binding site is innately stable despite considerable circumstantial evidence to the contrary[1–4]. However, the receptor structure (or subunit composition) is definitely perturbed by this concentration of urea. Analysis of liganded (labelled) receptor by HPSEC in 6 *M* urea results in a relatively broad elution profile with positive indications for the presence of at least 2–3 differently sized steroid-binding components, namely 31–32, 33–34 and 35 min.

Parallel analysis of unliganded (unlabelled) receptor presents a slightly altered elution profile with the major peak now eluting at 36–38 min. Even though a difference in retention time of 2–3 min is slight ($n = 3$), the altered general appearance of the HPSEC receptor elution profiles (in the presence and absence of steroid) would argue that this may indeed represent a steroid-dependent alteration in receptor structure. Furthermore, our data indicate that this technique is reproducible to within a 1 min variation in retention time. We are continuing to investigate urea effects on receptor structure (in the presence and absence of steroid) using sucrose density gradient centrifugation and high-performance size-exclusion columns with more potential for resolving receptor forms of intermediate size.

Steroid-dependent alterations in receptor structure become more distinct when receptor is analyzed by HPSEC in buffers containing 6 *M* urea and 0.4 *M* potassium chloride. Under these conditions, liganded receptor elutes in a distinctive, clearly altered manner. The profile is unlike that generated with either 6 *M* urea or 0.4 *M* potassium chloride and suggests that 6 *M* urea makes the majority of receptor (41–42 min) less sensitive to the dissociating effects of 0.4 *M* potassium chloride. The combined effects of 6 *M* urea and 0.4 *M* potassium chloride on receptor structure appear to be steroid-dependent. Under identical conditions, HPSEC of unliganded receptor results in an elution profile more like that seen when unliganded receptor is analyzed in 6 *M* urea alone. The presence of steroid makes the receptor more sensitive to the structure-dissociating effects of urea–potassium chloride, but still not as sensitive as the receptor in potassium chloride alone.

Individual HPSEC fractions of unliganded receptor may be sequentially analyzed (in the continued absence, or presence of steroid) to help delineate the sequence of steroid-dependent dissociation events and identify putative non-steroid-binding components of the native (unliganded) estrogen receptor protein. Since we have found both urea (up to 7.5 *M*) and 0.4 *M* potassium chloride effects to be reversible (data not shown) it may eventually be possible *in vitro* to fully dissect the individual components which constitute the potentially heterologous steroid–receptor protein complex.

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REFERENCES

1 M. R. Sherman, M. C. Moran, F. B. Tuazon and Y.-W. Stevens, *J. Biol. Chem.*, 258 (1983) 10366.
2 E.-M. Niu, R. M. Neal, V. K. Pierce, and M. R. Sherman, *J. Steroid Biochem.*, 15 (1981) 1.
3 M. R. Sherman and J. Stevens, *Ann. Rev. Physiol.*, 46 (1984) 83.
4 M. K. Dahmer, P. R. Housley and W. B. Pratt, *Ann. Rev. Physiol.*, 46 (1984) 67.
5 T. W. Hutchens, F. S. Markland and E. F. Hawkins, *Biochem. Biophys. Res. Commun.*, 103 (1981) 60.
6 T. W. Hutchens, F. S. Markland and E. F. Hawkins, *Biochem. Biophys. Res. Commun.*, 105 (1982) 20.
7 T. W. Hutchens, F. S. Markland and E. F. Hawkins, *Endocrinology*, 110 (1982) 221.
8 E. F. Hawkins, T. W. Hutchens, S. Fligiel, D. Horn and F. S. Markland, *J. Steroid Biochem.*, 16 (1982) 673.
9 T. W. Hutchens, W. E. Gibbons and P. K. Besch, *The 7th International Congress of Endocrinology, Quebec, 1984*, International Congress Series 652, Excerpta Medica, Amsterdam 1984, p. 756.

10 G. Redeuilh, C. Secco, E.-E. Baulieu and H. Richard-Foy, *J. Biol. Chem.*, 256 (1981) 11 496.
11 R. K. Puri and D. O. Toft, *Endocrinology*, 115 (1984) 2453.
12 P. Grandics, A. Miller, T. J. Schmidt, D. Mittman and G. Litwack, *J. Biol. Chem.*, 259 (1984) 3173.
13 T. W. Hutchens, H. E. Dunaway and P. K. Besch, *J. Chromatogr.*, 327 (1985) 247.
14 T. W. Hutchens, R. D. Wiehle, N. A. Shahabi and J. L. Wittliff, *J. Chromatogr.*, 266 (1983) 115.
15 T. W. Hutchens, W. E. Gibbons and P. K. Besch, *J. Chromatogr.*, 297 (1984) 283.
16 J. A. Katzenellenbogen, K. E. Carlson, D. F. Heiman, D. W. Robertson, L. L. Wei and B. S. Katzenellenbogen, *J. Biol. Chem.*, 258 (1983) 3487.
17 A. Munck and R. Foley, *Nature (London)*, 278 (1979) 752.
18 R. D. Markovic and G. Litwack, *Arch. Biochem. Biophys.*, 202 (1980) 374.
19 T. W. Hutchens, E. F. Hawkins and F. S. Markland, *J. Steroid biochem.*, 16 (1982) 705.
20 A. C. Notides and S. Nielsen, *J. Biol. Chem.*, 249 (1974) 1866.