Journal of Steroid Biochemistry, 1976. Vol. 7, pp. 185–197. Pergamon Press. Printed in Great Britain

CHROMATOGRAPHIC AND OTHER PROPERTIES OF THE ESTROGEN RECEPTORS FROM HUMAN MYOMETRIUM

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(*Received* 3 *April* 1975)

SUMMARY

In order to purify the specific estradiol-binding proteins present in human uterus, myometrial cytosol was fractionated according to a variety of techniques, most of which were chromatographic. Multiple receptor forms have been shown to exist. Changes in conformation and structure occur during storage of cytosol either in the frozen state or at 4°C over extended periods. One receptor form binds selectively to DNA. Sucrose gradient centrifugation analysis indicates the presence predominantly of 3–4 S species although 7–8 S forms have been encountered on occasions. As approximated by sedimentation analysis the molecular weights of these binding proteins are 45,000 and 160,000 respectively. The use of an estrone carboxymethyloxime-agarose derivative in affinity chromatography of the receptor proteins is described. A purification scheme utilizing this adsorbent after elimination of contaminating serum proteins has been outlined.

INTRODUCTION

The formation of estradiol–receptor complexes in target organs appears to be necessary to promote normal biosynthetic processes and tissue growth [1–3]. Major efforts to characterize these specific macromolecules have involved extracts obtained from rat and calf uterus [4–8] and only recently has increasing attention been given to the receptors from human myometrium [9–11]. In this laboratory the properties of the receptor-association constant, molecular weight, ligand specificity, the influence of pH, temperature, storage and degradative enzymes have all been studied using human myometrial cytosol [9, 12–18]. Contaminants in the preparations could easily have modified these characteristics. A point had been reached in our research program where further meaningful work was not possible without some degree of receptor purity. Research aimed at purification of the receptor was then initiated and a number of protein purification methods were assessed.

MATERIALS AND METHODS

Tissues and cytosol preparation. Human uterine tissue was obtained at hysterectomy and after removal was immediately put on ice. The tissues were immersed in four times their weight of ice-cold buffer, homogenized and the cytosol isolated as previously described [15]. If the cytosol was not used fresh it was frozen and stored at −20°C.

References in the text to "labelled cytosol" will indicate cytosol which had been incubated with $7{\cdot}2 \times 10^{-13}$ mol of [^{3}H]-estradiol (S.A. 46 Ci/mmol) per ml at 4°C over 4–16 h. Where indicated excess steroid was removed by adsorption to Dextran-coated charcoal as described below.

Steroids. [6,7-^{3}H]-Estradiol-17β (S.A. 46 Ci/mmol) and [2,4,6,7-^{3}H]-estradiol-17β (S.A. 93 Ci/mmol) were purchased from Radiochemical Centre, Amersham, England. The purity was checked by thin-layer chromatography.

Estradiol-17β and estrone were both obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A. while testosterone and 17β-estradiol 17-hemisuccinate were supplied by Steraloids Ltd., Croydon, Surrey, England.

Counting of radioactivity. Radioactivity was measured in a Tri-Carb model 3375 liquid scintillation spectrometer with automatic external standardization and coupled to a model 544 Absolute Activity Analyzer (Packard Instrument Co. Inc., Ill., U.S.A.). The counting error was always below 2·5%.

Chemicals. Dithiothreitol and phenylmethyl sulphonylfluoride were purchased from Calbiochem, Carlingford, N.S.W. Sephadex gels G-50, G-200 and Sepharose 4B were supplied by Pharmacia, Lane Cove, N.S.W. Ampholine pH 5–8 (40%) was obtained from L.K.B. Sweden. Miles–Yeda Ltd., Israel manufactured the poly-L-lysine hydrobromide used in affinity chromatography experiments.

Buffers. In these investigations the following buffers were used: 10 mmol Tris–HCl buffer, pH 8·0 (T_{10}, pH 8·0); 10 mmol Tris–HCl buffer, pH 7·4 (T_{10}, pH 7·4) and 10 mmol KH_2PO_4 adjusted to pH 7·4 with 1 M NaOH (10 mmol KH_2PO_4, pH 7·4). These

buffers, where indicated, contained appropriate quantities of EDTA (E), $CaCl_2$ (Ca), $MgCl_2$ (Mg), $MnCl_2$ (Mn), dithiothreitol (D), NaCl (Na) and KCl (K). In the remainder of the text all buffers will be given in their abbreviated form. For example 10 mM Tris–HCl buffer, pH 7·4 containing 200 mmol KCl, 1 mmol EDTA and 1 mmol dithiothreitol will be represented by $T_{10}K_{200}E_1D_1$, pH 7·4.

Dextran-coated charcoal suspensions were prepared by mixing 0·5% w/v charcoal (Norit A, Pfanstiehl, U.S.A.) and 0·1% w/v Dextran (Dextran T40, Pharmacia, Lane Cove, N.S.W.) in buffer.

All buffers were stored at 4°C and unless otherwise specified the experiments to be described were carried out at the same temperature.

Receptor binding assay. Generally estradiol receptor concentration was estimated by diluting an aliquot (usually 0·2, 0·5, or 1 ml) of sample to 1 ml with buffer and incubating with $2{\cdot}5 \times 10^{-13}$ mol of [^{3}H]-estradiol (S.A. 46 Ci/mmol) at 25°C for 30 min. The reaction mixture was then cooled in ice-water and 1 ml of a suspension of Dextran-coated charcoal in buffer was added. After brief mixing (Vortex), the mixture was kept in ice-water for 20 min after which time the solution was centrifuged at 4°C and 3500 rev./min for 10 min. The supernatant fraction was decanted into a scintillation vial, 10 ml of Bray scintillator [19] was added and the radioactivity was measured.

[^{3}H]-Estradiol-receptor concentration in labelled cytosol or fractions from chromatography experiments was determined by diluting an aliquot (0·2, 0·5 or 1 ml) to 1 ml with buffer followed by the Dextran-coated charcoal treatment mentioned above. Where indicated allowance was made for non-specifically bound [^{3}H]-estradiol. This was determined by heating an aliquot (0·2, 0·5 or 1 ml) at 60°C for 15 min, diluting to 1 ml with ice-cold buffer, followed by treatment with Dextran-coated charcoal in the usual way.

Estimation of protein concentration. The protein concentration was estimated by the method of Lowry *et al.* [20] based on the Folin-reaction. Protein elution profiles from filtration and chromatography experiments were traced from optical density measurements at 280 nm.

Ammonium sulphate precipitation. Finely powdered ammonium sulphate was added with stirring to labelled or unlabelled cytosol over a 30 min period to give a 30% saturated salt solution. After stirring for a further 30 min the precipitate was collected by centrifugation at 3500 rev./min for 20 min. The clear supernatant was decanted and the precipitate was dissolved in a small volume of buffer (usually 1/3 of original cytosol volume) to give a partially purified receptor extract.

Sephadex G-200 gelfiltration. Columns with Sephadex G-200 (bed vol. ~140 ml) were prepared in T_{10}, pH 8·0 and $T_{10}K_{200}E_1D_1$, pH 7·4 buffers. The void volume was determined with Blue Dextran 2000 (Pharmacia, Lane Cove, N.S.W.). Samples (5 ml) containing receptor-[^{3}H]-estradiol complex were applied to the columns and 3 ml fractions were collected at 5 ml/h. Aliquots were counted for ^{3}H estimation.

Hydroxyapatite chromatography. Columns (bed volume 50 ml) were prepared with 15g of hydroxyapatite (Bio-Rad, Richmond, Cal., U.S.A.) equilibrated in 10 mmol KH_2PO_4, pH 7·4 buffer. Sample application was followed by a 100 ml wash with the phosphate buffer and adsorbed protein was eluted with a linear gradient to 0·5 M KH_2PO_4 buffer adjusted to pH 7·4 with 1M NaOH. The KH_2PO_4 concentration in fractions of interest was estimated by reference to a concentration against volume graph which could be constructed by drawing a straight line between points indicating initial and limiting concentrations over the distance representing the total volume of the gradient.

DEAE-cellulose ion-exchange chromatography. Microgranular DEAE-cellulose (Whatman DE52, W. & R. Balston Ltd., Kent, England) was equilibrated in 10 mmol KH_2PO_4, pH 7·4 buffer. Samples prepared in this buffer were applied to packed columns followed by a wash with the same buffer. A linear gradient to 0·4 M KCl was then introduced. Aliquots from collected fractions were withdrawn for radioactivity measurement or determination of estradiol binding activity. The KCl concentration of the effluent was determined conductimetrically.

Preparation of DNA-cellulose. DNA-cellulose was prepared from calf-thymus DNA (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) and Whatman CF 11 cellulose (W. & R. Balston Ltd., Kent, England) according to methods described by Alberts and Herrick [21] and Litman [22]. The preparations contained 1–5 mg DNA/g cellulose as determined by acid digestion and Burton's colorimetric estimation of DNA [23].

Procedure for DNA-cellulose chromatography. DNA-cellulose (2·5 ml) was washed with 2 × 5 aliquots of $T_{10}D_1$, pH 7·4 buffer by mixing, centrifuging and then decanting the supernatant. Cytosol (usually 5 ml) prepared in $T_{10}D_1$, pH 7·4 was labelled with [^{3}H]-estradiol and then added to the washed DNA-cellulose pellet. Normally the mixture was incubated with frequent stirring at 25°C for 30 min or overnight at 4°C. After centrifugation the supernatant was decanted and the pellet was washed by repeating this procedure with 5 × 5 ml aliquots of the same buffer plus buffer containing 0·5 M KCl. Aliquots from each wash were withdrawn for radioactivity estimation. DNA-cellulose chromatography of partially purified receptor extracts was carried out in a similar way.

Sucrose gradient centrifugation. For sedimentation analysis, 0·2–0·3 ml samples were layered on 4·5 ml cold preformed [47] 5–20% sucrose gradients prepared in 10 mmol KH_2PO_4, pH 7·4 buffer containing 1 mM dithiothreitol and $T_{10}E_1D_1$, pH 7·4 with and without 0·5 M KCl. The gradients were centrifuged

for 16 h at 4°C and 140,000 *g* in a M.S.E.-75 Ultracentrifuge and 10 drop fractions were collected through the bottom of the tubes. γ-Globulin and albumin, used as sedimentation markers were supplied by Serva (Germany) and sucrose for gradient centrifugation was produced by Merck (Germany).

Electrofocusing. Samples for electrofocusing were prepared in 1 mmol KH_2PO_4, pH 7·4 buffer by filtering an aliquot (up to 15 ml) through a column of Sephadex G-50 (bed vol. 50 ml) equilibrated in this buffer. The experiments were carried out in a 110 ml standard electrofocusing column (LKB, Sweden) over 20–24 h at 4°C and 400 V. A pH 5–8 gradient was achieved using 1·5% ampholine in a 0–50% sucrose gradient. Samples were added to the column during the manual formation of the sucrose gradients. Fractions (3 ml) were collected and the pH and radioactivity in each were measured.

Con A-Sepharose chromatography. Con A-Sepharose (Pharmacia, Lane Cove, N.S.W.) was equilibrated in $T_{10}Na_{500}Ca_1Mg_1Mn_1D_1$, pH 7·4 buffer and packed in a column to give a 50 ml bed vol. Labelled or unlabelled cytosol (30–40 ml) prepared in the same buffer, was applied to the column followed by a 150 ml wash with buffer. Protein bound to the column was eluted with buffer containing 2% glucopyranoside (Calbiochem, Carlingford, N.S.W.). Aliquots (0·5 ml) from 10 ml fractions were assayed for receptor-[^{3}H]-estradiol complex or for estradiol binding activity. Radioactivity was counted using toluene scintillator (6 g PPO, 75 mg POPOP in 1000 ml toluene; 10 ml of scintillator to 1 ml of aq. sample). Partially purified receptor extracts were chromatographed in the same way. The Con A-Sepharose was reactivated ready for use by washing with 500 ml of $T_{10}Na_{500}Ca_1Mg_1Mn_1D_1$, pH 7·4 buffer.

Preparation of estrone 17-(O-carboxymethyl)oxime. A mixture of estrone (100 mg) and O-carboxymethyl hydroxylamine hydrochloride (100 mg) (Calbiochem, Carlingford, N.S.W.) was dissolved in a solution containing 20% (v/v) aq. methanol (12 ml) and 1M sodium acetate (8 ml). The reaction mixture was stirred at room temperature overnight and after transferring to a separatory funnel 200 ml of saturated $NaHCO_3$ was added. The mixture was washed twice with ether and then acidified (HCl). Extraction of the organic material with ethyl acetate and evaporation of the dried solvent gave the carboxymethyloxime (95 mg). Recrystallization from acetone afforded needles m.p. 186–187°C.

Synthesis and washing of estrone 17-(O-carboxymethyl)oxime-poly(L-Lysine)agarose. Estrone 17-(O-carboxymethyl)oxime-poly(L-lysine)-agarose was prepared by adapting synthetic methods described by Sica *et al.* [24]. The gel (10 ml) was washed on a Buchner funnel with 2 l of dioxan over 2 days, 2 l of 80% methanol (2 days) and finally 500 ml of distilled water (1 h).

Before use in purification experiments the adsorbent was diluted 5 fold with Sepharose 4B and the mixture was washed with dioxan (500 ml for 10 ml of gel), 90% methanol (500 ml) and water (500 ml) over 4–6 h.

Stability testing of affinity adsorbent. Breakdown of the estrone carboxymethyloxime-agarose derivative under the slightly alkaline conditions (pH 7·4) used during chromatography could lead to leakage of estrone 17-(O-carboxymethyl)oxime or estrone into the incubating medium. Theoretically hydrolysis could occur at the carbon nitrogen double bond (to give estrone) or at the amide linkage (giving the oxime).

Checks on hormone leakage after incubation with cytosol were carried out as follows: The gel was separated from the cytosol by centrifugation and an aliquot (5 ml) of supernatant was extracted with ether (2 × 10 ml). Evaporation of the dry ethereal extract gave a residue which was dissolved in 200 μl of dimethylformamide–water (1:1, v/v). Aliquots (20 μl) of this solution, together with $2·5 \times 10^{-13}$ mol of [^{3}H]-estradiol were incubated with 0·5 ml of cytosol. Cytosol not exposed to the affinity adsorbent was treated in identical manner and served as the control. Binding inhibition was measured by comparison of the binding in both instances. Although [^{3}H]-estradiol was used in these competition studies perhaps a more sensitive test of affinity column stability would have been provided by using [^{3}H]-estrone.

Affinity chromatographic procedure. Frozen cytosol (150 ml), accumulated from a number of patients, was thawed and subjected to ammonium sulphate precipitation. After centrifugation the pellet was dissolved in 45 ml of $T_{10}K_{200}E_1D_1$, pH 7·4 buffer and stirred with 9 ml of adsorbent at 4°C overnight. The mixture was transferred to a column and after filtration had occurred a wash with 200 ml of $T_{10}K_{200}E_1D_1$, pH 7·4 and 100 ml of $T_{10}K_{1000}E_1D_1$, pH 7·4 was applied. The adsorbent was then transferred to a Buchner funnel and washed with a further 100 ml of the 200 mmol KCl buffer. The adsorbent cake containing receptor was put into a conical flask containing 20 ml of $T_{10}K_{200}E_1D_1$, pH 7·4 buffer with 0·97 μmol [^{3}H]-estradiol (S.A. 5·14 Ci/mmol) and the suspension was incubated at 30°C for 15 min with occasional swirling. This was followed by a rapid filtration into an ice-cold filtering flask through a warm (~ 30°C) Buchner funnel. The gel was rinsed with 5 ml of warm (30°C) $T_{10}K_{200}E_1D_1$, pH 7·4 buffer and the combined filtrate was left standing at 4°C overnight.

Assay for receptor-[^{3}H]-estradiol complex recovered from affinity chromatography. This followed the hydroxyapatite method of receptor estimation which has been described by Erdos *et al.* [25], in which extracts containing receptor-[^{3}H]-estradiol complex are adsorbed on 0·5 ml hydroxyapatite columns. Free hormone is rapidly eluted with buffer at 4°C while the receptor–steroid complex is retained on the column. Retained radioactivity is measured after quantitative transfer of the hydroxyapatite into counting vials. A portion (5 ml) of the filtrate recovered

from affinity chromatography was desalted on a Sephadex G-50 column (bed vol. 50 ml) equilibrated in 1 mmol KH_2PO_4, pH 7·4 buffer containing 1 mmol dithiothreitol. The fractions containing macromolecular bound radioactivity were combined (10 ml) and duplicate 0·5 ml aliquots were passed through 0·5 ml hydroxyapatite columns followed by a 15 ml wash with the phosphate buffer. The hydroxyapatite was ejected into vials, 15 ml of Bray scintillator was added and the mixture was vigorously shaken. The vials were centrifuged and radioactivity was counted in the usual way. The level of non-specific binding was estimated by heating a 3 ml aliquot of the combined fractions at 60°C for 15 min and repeating the procedure.

Estimation of protein recovered from affinity chromatography. The protein content in the filtrate was determined using fluorescamine [26] (Roche Products Pty., Dee Why, N.S.W.). The filtrate (1·5 ml) was passed through a Sephadex G-50 column (bed vol. 50 ml) equilibrated in 0·2 M boric acid–sodium hydroxide buffer, pH 9·2 and 2 ml fractions were collected. Fluorescamine reagent was added to fractions 1–20 in 0·5 ml aliquots. Standards were made up in triplicate by adding known protein quantities (0·5–20 μg) to blank fractions obtained from the column.

RESULTS

Ammonium sulphate precipitation. At 30% ammonium sulphate saturation some selective precipitation of the receptor–estradiol complex from labelled human myometrial cytosol was observed. Purifications of 2 fold with 60% recoveries were consistently obtained. Residual receptor–estradiol complex concentrations in the supernatants could not account for the 40% loss. Neither could this loss be remedied to any great extent by increased salt concentration of the dissolving medium nor by increasing the amount of ammonium sulphate added to the cytosol. The inclusion of divalent cations (Ca, Mg, Mn) also had no influence. The losses must therefore be due to dissociation and denaturation effects.

Sephadex G-200 gelfiltration. Gelfiltration of labelled cytosol on Sephadex G-200 revealed two peaks of radioactivity representing components having relative elution volumes of 1·91 (peak 1) and 2·54 (peak 2) using Dextran Blue 2000 as reference (Fig. 1). The receptor–estradiol complex was localized to peak 1 by incubating aliquots from the fractions with Dextran-coated charcoal to distinguish between receptor bound [^{2}H]-estradiol and non-specifically bound or free hormone. Peak 2 showed no retention of radioactivity.

Density gradient centrifugation. Sucrose gradient sedimentation analysis of human myometrial cytosol repeatedly showed bound [^{3}H]-estradiol sedimenting at about 3·5S. A 7S binding component was less frequently observed and appeared to be unstable during storage (Fig. 2). It was possible that proteolytic

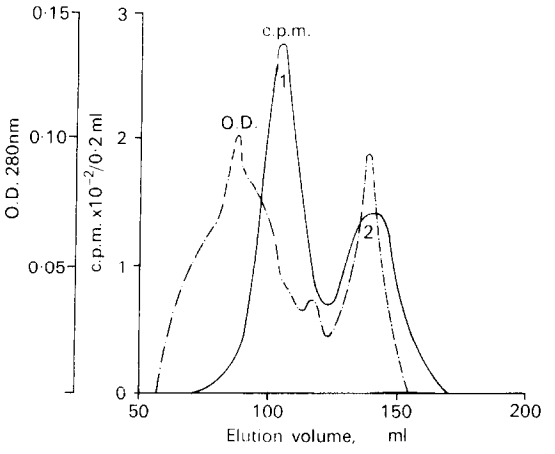

Fig. 1. Elution profile of labelled cytosol on Sephadex G-200. Labelled cytosol (5 ml), prepared in T_{10}, pH 8·0 buffer was filtered through a column of Sephadex G-200 (bed vol. 140 ml) packed in the same buffer. Fractions (3 ml) were collected and measured for radioactivity content and absorbance at 280 nm.

enzymes present in the cytosol were degrading the 7S receptor to the smaller binding unit [10, 45] but addition of the highly effective protease inhibitor phenylmethyl sulphonylfluoride [46] in 1 mmol concentration afforded no protection (Fig. 2). No trace of the 7S binding component was present after storage of the same labelled cytosol at 4°C over 7 days with or without protease inhibitor even in the presence of dithiothreitol (Fig. 2b).

Based on density gradient analysis and by comparison to the sedimentation behaviour of standards (human serum γ-globulin, 7S, molecular weight 160,000 and egg albumin, 3·6S, molecular weight 45,000) the molecular weights of the 7S and 3·5S receptors are in the vicinity of 160,000 and 45,000 respectively.

Hydroxyapatite chromatography. Fractionation of human myometrial cytosol on hydroxyapatite was also of interest. By elution with a linear KH_2PO_4 gradient two peaks of radioactivity were produced, both of which were accompanied by 280 nm absorbing material (Fig. 3). The retained radioactivity (peak 2), eluted with 0·1 M KH_2PO_4 buffer, pH 7·4, was shown to be receptor bound by Dextran-coated charcoal treatment. Albumin has been shown in this laboratory to elute from hydroxyapatite in the same region as the receptor-[^{3}H]-estradiol complex.

Ion-exchange chromatography. In our experience fractionation of labelled cytosol on DEAE-Sephadex A-50 ion-exchange resin proved unsatisfactory. The receptor-[^{3}H]-estradiol complex was repeatedly eluted from these columns over the whole extent of the linear KCl gradient used as elution medium.

Poor resolution between receptor–steroid complex and other proteins also occurred when cytosol was chromatographed on DEAE-cellulose (DE52) equilibrated in Tris buffers. A much better separation was obtained on equilibration in 10 mmol KH_2PO_4, pH

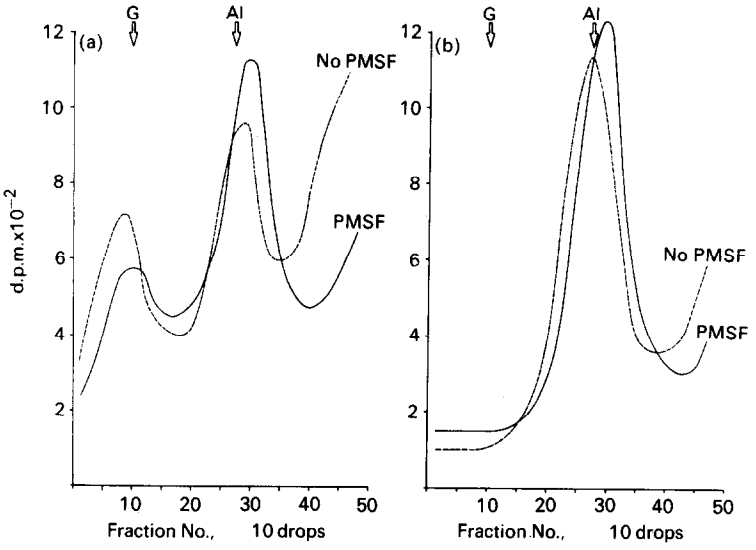

Fig. 2. Sedimentation analysis of estradiol-binding preparations. Cytosols and sucrose gradients (5–20%) were prepared in 10 mmol KH_2PO_4 buffer, pH 7·4 containing 1 mmol dithiothreitol and 5% isopropanol with or without 1 mmol phenylmethyl sulphonylfluoride (PMSF). A: Excess [^{3}H]-estradiol was removed from labelled cytosols with Dextran-coated charcoal and 200 μl aliquots were layered on the gradients. Centrifugation took place over 16 h at 4°C and 140,000 *g*. B: After 7 days storage at 4°C the same cytosols were analysed in a similar way. Sedimentation markers: G, γ-globulin (7S) and Al, egg albumin (3·6S).

7·4 buffer and as illustrated in Fig. 4 at least two binding components (peaks 1 and 2) were evident. KCl elution molarities were zero for peak 1 and approximately 0·05 M for peak 2 (Fig. 4). The binding of [^{3}H]-estradiol by both components was inhibited by non-radioactive estradiol, but not by testosterone, indicating some degree of specificity. Both proteins were shown to have similar molar association constants ($\sim 1 \times 10^9$ M^{-1}) for the interaction with estradiol.

In our laboratory it has been found that 1 mmol dithiothreitol [27] prevents the deterioration of estradiol binding in human myometrial cytosol with storage [17] suggesting the importance of sulphydryl groups in the receptor. The protective reagent is now used with all receptor preparations. During ion-exchange chromatography of labelled cytosol some dissociation of the receptors from estradiol was anticipated giving rise to fractions containing both receptor-[^{3}H]-estradiol complex and free receptor. Subsequently, it was shown by chromatographic treatment of labelled cytosol in the presence and absence of dithiothreitol that the reagent effectively protected the free receptor sites (Fig. 5). On this occasion, in addition to the peaks 1 and 2 previously encountered (Fig. 4), a third peak of radioactivity (peak 3) eluting at a slightly higher KCl molarity was noted (Fig. 5). This could be attributed to a more aggregated receptor

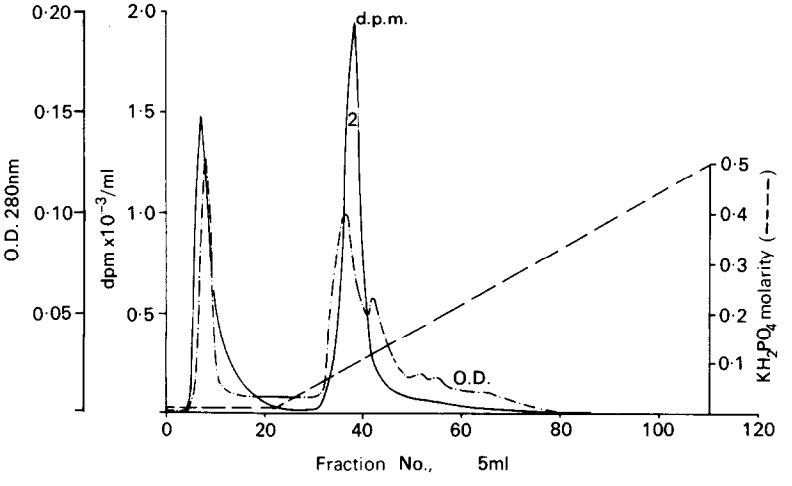

Fig. 3. Elution profile of labelled cytosol on hydroxyapatite. Labelled cytosol (5 ml), prepared in 10 mmol KH_2PO_4 buffer, pH 7·4 was applied to a hydroxyapatite column (bed vol. 50 ml) packed in the same buffer. Retained proteins were eluted with a linear gradient to 0·5 M KH_2PO_4 over 450 ml.

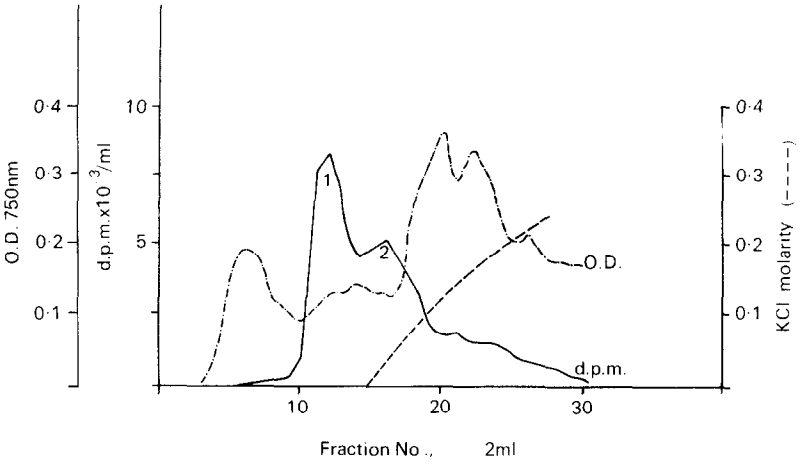

Fig. 4. Elution profile of labelled cytosol on DEAE-cellulose. Free or loosely bound [^{3}H]-estradiol was removed by charcoal adsorption from labelled cytosol (5 ml) prepared in 10 mmol KH_2PO_4, pH 7·4 buffer. The cytosol was applied to a DEAE-cellulose column (bed vol. 8 ml) equilibrated in the phosphate buffer and elution was carried out with a linear gradient to 0·4 M KCl over 90 ml. Fractions (2 ml) were collected and 1 ml aliquots were counted for ^{3}H. Protein content in each fraction was determined on 0·1 ml aliquots according to the Lowry method.

form although no other evidence was obtained to support this view. As expected Dextran-coated charcoal treatment caused a reduction in the apparent receptor-bound radioactivity although this decrease was more pronounced without dithiothreitol (Fig. 5). Furthermore on reincubation of the eluted fractions with [^{3}H]-estradiol and elimination of the unbound hormone by exposure to Dextran-coated charcoal, it was revealed that peaks 2 and 3 in the stabilized case achieved 100% reassociation to the original level (Fig. 5b). In contrast the corresponding fractions from the untreated cytosol exhibited reassociation to levels only slightly above those obtained after Dextran-coated charcoal incubation (Fig. 5a). No restoration of complex was observed with peak 1 fractions in either experiment possibly indicating irreversible dissociation.

DNA-cellulose chromatography. Receptor-[^{3}H]-estradiol complex from human myometrial cytosol binds optimally to DNA-cellulose on incubation at 4°C overnight or 25°C for 30 minutes (Fig. 6). The complex is recovered by washing with 0·5 M KCl. Although some results were obtained without the use of optimum conditions purifications of 5–30-fold with up to 50% recoveries have been observed. Incubation at 37°C for 15 min gave reduced binding probably due to denaturation effects. Other results indicate the specific nature of the receptor–hormone complex-DNA interaction (Fig. 7). Receptor not previously treated with labelled steroid also binds to the DNA-cellulose adsorbent, but estradiol increases the affinity of the receptor for DNA (Fig. 7). [^{3}H]-Estradiol bound to either steroid binding β-globulin (obtained from pregnancy plasma) or human serum albumin was not retained to any detectable extent. This negates the possibility that a high affinity complex between [^{3}H]-estradiol and any protein may be indiscriminately bound to DNA-cellulose.

The influence of ionic strength on the interaction was also investigated. Our results indicated that maximal binding occurs with zero KCl concentration and a decreased binding tendency was observed with increased salt concentration to 0·3 M.

In all our experiments with DNA-cellulose total binding of the applied receptor-[^{3}H]-estradiol complex was never observed. Different cytosols with similar complex concentrations exhibited greatly different binding properties. A difference was also apparent between fresh and stored (−20° or 4°C) cytosols where binding to DNA-cellulose decreased with storage even though receptor–hormone complex concentration remained unchanged. Partial purification of receptor present in frozen, stored cytosols by ammonium sulphate precipitation did not enhance binding.

Initially it was thought that uptake of the receptor-[^{3}H]-estradiol complex might be limited by insufficient DNA being bound to the cellulose (1 mg DNA/g cellulose). An alternative method of preparation [22] gave higher yields—5 mg/g on small scale and 2·6 mg/g on large scale preparation. Equal weights of all DNA-cellulose adsorbents with DNA concentrations of 1 (DNA_1), 2·6 (DNA_2) and 5 (DNA_3) mg/g were tested for their ability to bind receptor-[^{3}H]-estradiol complex from fresh cytosol. Untreated cellulose was run as a control. The results (Table 1) showed that increased DNA adsorption did not increase the efficiency of receptor binding to the adsorbents and suggested that the capacity to bind DNA-cellulose was cytosol dependent. The cytosol might contain receptors which have different conformations only one of which is conducive to DNA binding.

As mentioned earlier chromatography of cytosol on DEAE-cellulose ion-exchange resin revealed a multiplicity of peaks and this technique was used to study

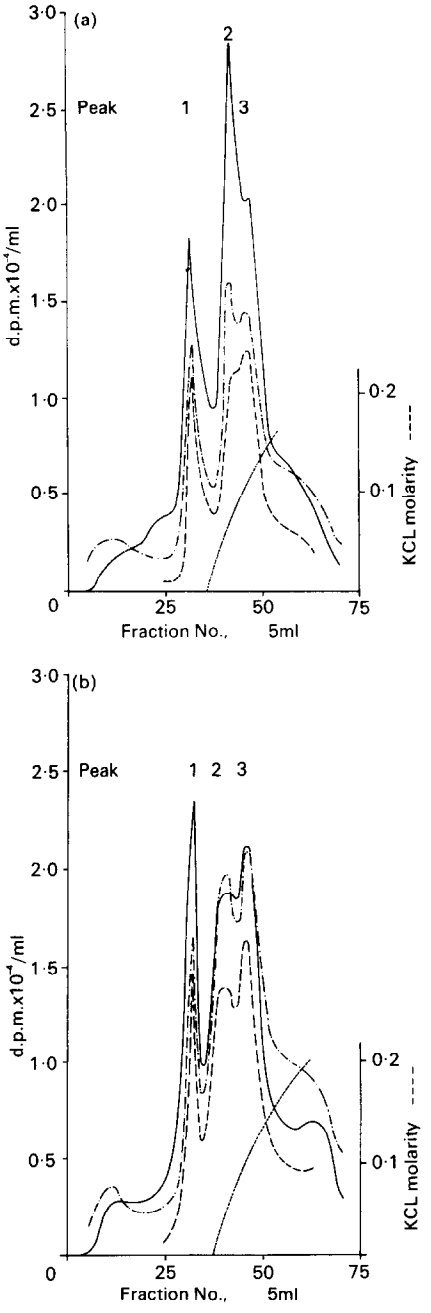

Fig. 5. Effect of dithiothreitol on receptor stability during DEAE-cellulose chromatography. A: Chromatography with no dithiothreitol. Cytosol (20 ml), prepared in 10 mmol KH_2PO_4, pH 7·4 buffer, was labelled with [^{3}H]-estradiol and applied to a DEAE-cellulose column (1·6 × 30 cm., bed vol. 50 ml) packed in the same buffer. Retained proteins were eluted in 5 ml fractions with a linear gradient over 300 ml to 0·4 M KCl. B: Chromatography with dithiothreitol (1 mmol) throughout. The stabilized cytosol was treated as in A. In both experiments 1 ml aliquots were taken from fractions for ^{3}H measurement (——) and residual radioactivity, after exposure of 1 ml aliquots to Dextran-coated charcoal, was also determined (– – –). A further series of 1 ml portions were incubated with $2·5 \times 10^{-13}$ mol of [^{3}H]-estradiol (S.A. 46 Ci/mmol) and then with the same charcoal buffer to determine receptor bound radioactivity (—·—).

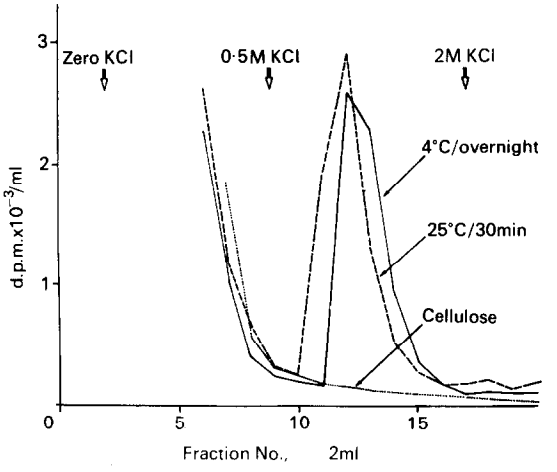

Fig. 6. Binding of receptor-[^{3}H]-estradiol complex to DNA-cellulose. Fresh labelled cytosol (3 ml) in $T_{10}K_{50}E_1D_1$, pH 7·4 buffer was incubated with DNA-cellulose (2·5 ml) at 25°C for 30 min and at 4°C overnight with occasional stirring. The mixtures were transferred to 0·9 cm. dia. columns and the packed adsorbent was successively eluted with 15 ml each of $T_{10}K_{50}E_1D_1$, pH 7·4; $T_{10}K_{500}E_1D_1$, pH 7·4 and $T_{10}K_{2000}E_1D_1$, pH 7·4 buffers. Fractions (2 ml) were collected and 1 ml aliquots were withdrawn for radioactivity determination. Labelled cytosol incubated with cellulose at both temperatures served as a control.

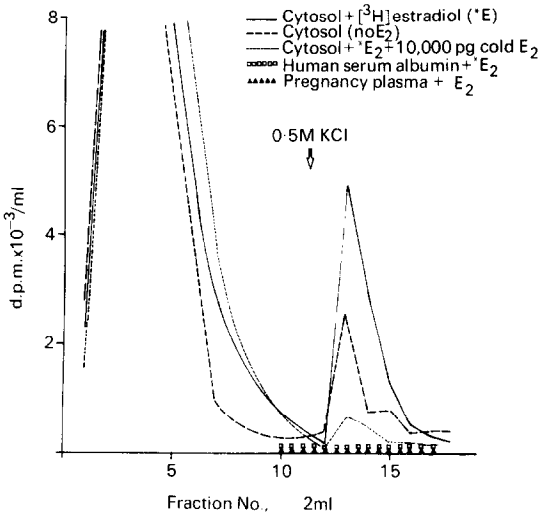

Fig. 7. Specificity of DNA-cellulose adsorption. DNA-cellulose (2·5 ml) was incubated at 4°C overnight with 3 ml of fresh cytosol (B); fresh, labelled cytosol (A) and fresh cytosol labelled with [^{3}H]-estradiol (*E_2) in the presence of 10,000 pg estradiol (E_2) (C). The mixtures were transferred to columns and washed successively with 15 ml each of $T_{10}K_{50}E_1D_1$, pH 7·4 and $T_{10}K_{500}E_1D_1$, pH 7·4 buffers. Aliquots (1 ml) from 2 ml fractions were measured for radioactivity (A, C) or estradiol binding activity (B, binding capacity expressed as d.p.m./ml). Similar experiments were carried out with solutions containing human SA and steroid binding β-globulin (protein concentration approximately 5 mg/ml in both cases) after both had been incubated with *E_2 at 4°C overnight.

Table 1. Influence of DNA concentration on receptor-DNA-cellulose interaction. Aliquots (5 ml) of fresh, labelled cytosol prepared in $T_{10}D_1$, pH 7·4 buffer were incubated at 4°C overnight with 2·5 ml of DNA-cellulose having varying DNA content: DNA_1 (1 mg DNA/g cellulose), DNA_2 (2·6 mg/g), DNA_3 (5 mg/g). Untreated cellulose was used as the control. Each adsorbent was washed successively with 5 × 5 ml $T_{10}D_1$, pH 7·4 and 5 × 5 ml $T_{10}K_{500}D_1$, pH 7·4 buffers by alternatively centrifuging and decanting the supernatants. Receptor-[^{3}H]-estradiol complex (R-*E_2) present in the $T_{10}D_1$, pH 7·4 wash (R-*E_2 not retained) and in the $T_{10}K_{500}D_1$, pH 7·4 wash (R-*E_2 retained) was quantitated

Adsorbent	DNA conc.	R-*E_2 Applied	R-*E_2 Retained	% Total	R-*E_2 Not retained	% Total
	mg/g	d.p.m.	d.p.m.		d.p.m.	
DNA_1	1	180,000	86,000	48	80,000	45
DNA_2	2·6	180,000	88,000	49	64,000	36
DNA_3	5	180,000	86,000	48	92,000	51
Cellulose	0	180,000	zero	—	137,000	76

the receptor-[^{3}H]-estradiol-DNA-cellulose interaction. Fresh, labelled cytosol was incubated with DNA-cellulose and the adsorbent was washed with $T_{10}D_1$, pH 7·4 and $T_{10}K_{500}D_1$, pH 7·4 buffers. These washings as well as control cytosol not treated with DNA-cellulose were separately chromatographed on ion-exchange columns under identical conditions. The same labelled cytosol was stored at 4°C over 5 days, treated with DNA-cellulose and only the control was analyzed by ion-exchange chromatography. The results are shown in Fig. 8. Two peaks were evident in the fresh control cytosol (Fig. 8a). Ion-exchange chromatography of the proteins not retained by DNA-cellulose ($T_{10}D_1$, pH 7·4 wash) showed a reduction in peak 2 relative to peak 1 size suggesting that the former was being bound by the DNA-cellulose. This was confirmed when the 0·5 M KCl wash from the DNA-cellulose column was chromatographed to produce only peak 2. The 5 day stored cytosol, with receptor-[^{3}H]-estradiol complex concentration similar to that in the fresh cytosol, showed a marked decrease in binding to DNA (3% as against 15% by the fresh cytosol) and also gave the two peaks (Fig. 8b). Peak 2 was greatly diminished in intensity relative to peak 1, but whether it was being transformed to the peak 1 component could not be ascertained.

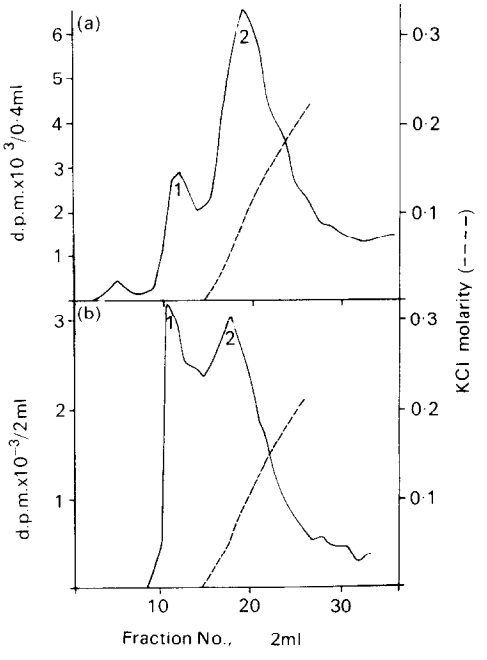

Fig. 8. Ion-exchange profiles of estradiol-receptor extracts used for DNA-cellulose adsorption experiments. Prior to chromatography on DEAE-cellulose the labelled samples were treated with a suspension of Dextran-coated charcoal to remove free or loosely bound [^{3}H]-estradiol. After equilibration in 10 mmol KH_2PO_4 buffer, pH 7·4 they were applied to DEAE-cellulose columns (bed vol. 8 ml) and retained proteins were eluted with a linear gradient to 0·4 M KCl over 90 ml. A: Control cytosol not treated with DNA-cellulose; B: Cytosol (identical to A) stored at 4°C over 5 days.

Sedimentation analyses on the fresh and stored control cytosols were carried out with 5–20% sucrose gradients in $T_{10}E_1D_1$, pH 7·4 and in $T_{10}K_{500}E_1D_1$, pH 7·4 buffers (Fig. 9). The fresh cytosol in the low salt buffer showed radioactivity sedimenting in the 7–8S and 3–4S regions (Fig. 9). In the high salt buffer, however, the faster sedimenting peak had disappeared and radioactivity was concentrated around the 4S region appearing as one peak with a shoulder (Fig. 9). The stored cytosol showed no 7–8S peak in low

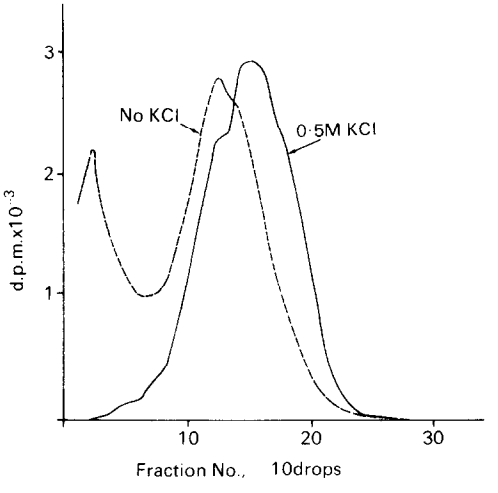

Fig. 9. Sedimentation patterns of cytosols before DNA-cellulose treatment. Labelled samples were treated with a suspension of Dextran-coated charcoal to remove free or loosely bound [^{3}H]-estradiol. Fresh, labelled cytosol (0·3 ml) was layered on 5–20% sucrose gradients prepared in $T_{10}E_1D_1$, pH 7·4 and $T_{10}K_{500}E_1D_1$, pH 7·4 buffers. Centrifugation took place at 140,000 *g* over 16 h at 4°C.

salt buffer, but in the high salt gradient the sedimentation pattern looked very similar to that obtained with fresh cytosol. These results with the stored cytosol suggest that the proteins represented by peaks 1 and 2 observed with ion-exchange chromatography, both exist as 4S components. An attempt to characterize the receptor-[^{3}H]-estradiol complex which had been retained on DNA-cellulose was unsuccessful because of insufficient radioactivity.

A similar experiment was designed to reproduce these results. The only variation was that the cytosol was frozen overnight and then thawed instead of being stored over 5 days at 4°C. Approximately the same quantities of receptor-[^{3}H]-estradiol complex were applied to the DNA-cellulose columns. Only 6·5% was bound by the column with the fresh cytosol as against 0·5% with the frozen, thawed cytosol. Analysis of the ion-exchange profiles showed that the fresh cytosol had only the peak 2 component. In contrast, both peak 1 and 2 components were shown to be present in the frozen/thawed cytosol and among the proteins not retained by DNA-cellulose on incubation with fresh cytosol. The distribution of radioactivity in these profiles suggested that peak 1 was being produced at the expense of peak 2.

Con A-Sepharose chromatography. Con A-Sepharose (concanavalin A covalently bound to Sepharose 4B) is a group specific adsorbent for polysaccharides and glycoproteins. The binding site of concanavalin A is specific for α-D-mannosyl, α-D-glucosyl and sterically similar residues. These residues are widely distributed in carbohydrate containing biopolymers. Elution of bound substances is carried out using either α-D-methylmannoside or α-D-methylglucoside [28–30].

The inactivation of the estrogen receptor from human uterus by enzymes such as β-glucosidase, β-galactosidase, α-glucosidase, amyloglucosidase, β-glucuronidase, neuraminidase and α-amylase indicated the presence of a carbohydrate moiety in the molecule [18]. It was of interest therefore to examine the behaviour of the receptor towards Con A-Sepharose since specific retention would provide partial purification.

Initial chromatography both of labelled and unlabelled cytosol on Con A-Sepharose showed retention of estradiol-binding proteins (Fig. 10). However, a high degree of estradiol-binding activity was always present in early fractions prior to elution with glucopyranoside (peak 1) and it was possible that contaminating glycoproteins were binding preferentially to the concanavalin A. Subsequently even with increased column size and partial purification of the receptor (ammonium sulphate precipitation and DEAE-cellulose chromatography) no alteration in this pattern occurred.

Characterization, by Scatchard plot analysis, of the estradiol-binding proteins present in the fractions eluted with and without glucopyranoside as well as the control cytosol not treated with Con A-Sepharose

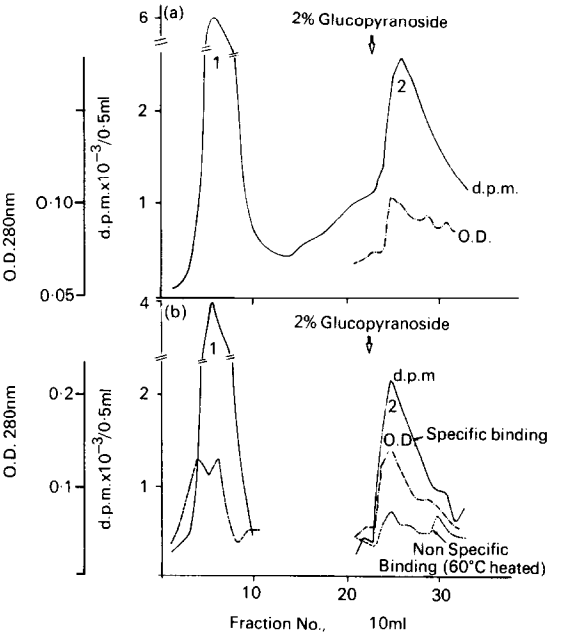

Fig. 10. Con A-Sepharose chromatography of labelled (A) and unlabelled (B) cytosol. Myometrial cytosol (30 ml) was applied to Con A-Sepharose packed (bed vol. 50 ml) in $T_{10}Na_{500}Ca_1Mg_1Mn_1D_1$, pH 7·4 buffer. After washing the column with 150 ml of buffer retained protein was eluted with 2% glucopyranoside in buffer. Aliquots (0·5 ml) were withdrawn from 10 ml fractions to determine ^{3}H content (A) or estradiol binding activity (B, binding capacity expressed as d.p.m./0·5 ml). With unlabelled cytosol (B) nonspecific binding in fractions of interest was measured after heating 0·5 ml aliquots at 60°C.

was carried out. Molar association constants were calculated and these are shown in Table 2. The retained estradiol-binding protein (Fig. 10, peak 2) had an association constant about 10 times less than that of the binding protein which was not reactive to concanavalin A (Fig. 10, peak 1). The control association constant was also lower in each case. Reported values [31, 32] for the association constant of steroid binding β-globulin with estradiol were similar to those obtained with the Con A-Sepharose retained protein. The identity of this protein as the β-globulin was supported by chromatography of human pregnancy plasma, previously incubated with [^{3}H]-estradiol, on Con A-Sepharose. Fractions having large amounts of radioactivity were observed on elution with buffer containing glucopyranoside. Confirmation that the fraction eluted with glucopyranoside was steroid binding β-globulin was obtained when testosterone was shown to inhibit [^{3}H]-estradiol binding. No inhibition occurred with the peak 1 fractions indicating that they contained the estradiol receptors.

Affinity chromatography. In this laboratory work with the affinity adsorbent 17β-estradiol-17-hemisuccinyl-poly(L-lysine)-agarose (Fig. 11a), described by Sica *et al.* [24, 33] in the purification of calf uterus receptor, showed that the adsorbent was unstable

Table 2. Characterization of estradiol-binding proteins by Scatchard plot analysis before and after Con A-Sepharose chromatography. Three fresh cytosols were examined. Aliquots (0·5 ml) from fractions eluted with (retained) and without (breakthrough) glucopyranoside and control cytosol not treated with Con A-Sepharose were tested for the influence of increasing concentrations of non-radioactive estradiol (50, 100, 200, 1000, 5000 pg) on the binding of [^{3}H]-estradiol (66 pg). Molar association constants in each case were calculated from Scatchard plots of these results

Cytosol number	Molar association constant of estradiol binding proteins present in: Control	Breakthrough fraction	Retained fraction
1	$1{\cdot}4 \times 10^9$	$1{\cdot}6 \times 10^{10}$	$1{\cdot}7 \times 10^9$
2	$5{\cdot}8 \times 10^9$	$1{\cdot}7 \times 10^{10}$	$1{\cdot}8 \times 10^9$
3	$2{\cdot}3 \times 10^{10}$	$2{\cdot}8 \times 10^{10}$	$2{\cdot}8 \times 10^9$

during chromatography of human myometrial cytosol. Even though extensive washing was carried out prior to use further release of estradiol was still observed. This was evidenced by significant binding inhibition observed when ethereal extract residues of supernatants exposed to the affinity adsorbent were incubated with fresh cytosol in the presence of [^{3}H]-estradiol. Consequently it was necessary to look for other less labile substrates.

A likely candidate was the 17-(*O*-carboxymethyl)oxime derivative of estrone which possesses the crucial A-ring structure for receptor binding [15] as well as a means of attachment to an insoluble matrix through the carboxy group. The substrate was readily synthesized from estrone and was shown to compete with estradiol for receptor binding sites only at high concentrations. Reciprocal plot analysis using 1 μg of oxime indicated that its affinity for the human myometrial receptor was almost 20,000 times less than that of estradiol. Estrone 17-(O-carboxymethyl)oxime poly(L-lysine)-agarose (Fig. 11b) was then synthesized according to established methods [24] and after extensive washing was diluted ten, five and two fold with Sepharose 4B. The binding capacity of the cytosol with which they were incubated was decreased by 50, 55 and 78% respectively. No release of substrate was apparent from inhibition tests using the ether extraction procedure described in the methods section. The stability of the adsorbents to storage in distilled water at 4°C was further demonstrated when periodical inhibition tests over 9 weeks showed only 5·5% binding inhibition. However, it was decided, as an added precaution, to wash the gel routinely with dioxan–methanol (1:1, v/v), 80% methanol and water before equilibration in buffer for use in purification experiments. The overall results of receptor extract chromatography on the affinity adsorbents are summarized in Table 3. A 1600 fold purification was achieved over starting cytosol. Recovery of the receptor from the adsorbent was 52%.

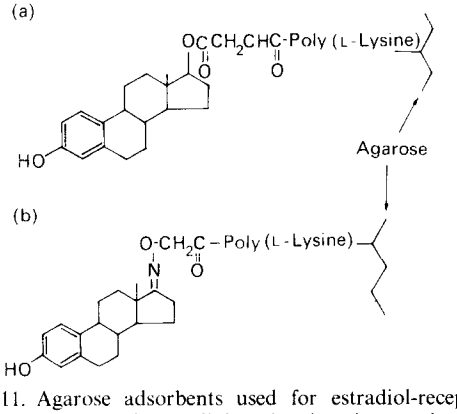

Fig. 11. Agarose adsorbents used for estradiol-receptor purification. A: 17β-Estradiol 17-hemisuccinate-poly(L-lysine)-agarose, B: estrone 17-(O-carboxymethyl)oxime-poly-(L-lysine)agarose.

Characterization of recovered receptor-[^{3}H]-estradiol complex by Sephadex G-200 chromatography produced confirmatory evidence for receptor presence. Samples from original cytosol mixture, the dissolved pellet from ammonium sulphate precipitation and the recovered extract both heated (60°C) and unheated were chromatographed on the same column. Except for the heated sample, in which the receptor–hormone complex would have been destroyed, each showed a peak of bound radioactivity at a relative elution volume of 1·75 (Fig. 12, peak 2). Aggregates were also evident. Isoelectric focusing afforded a major peak of radioactivity at pH 6·5 and two others at 5·8 and 5·6 (Fig. 13). In addition a large peak of radioactivity was observed in early fractions (pH < 5). Truong *et al.* [34] have obtained a similar pattern with calf uterus receptor recovered from an affinity column based on an agarose 7α-estradiol derivative.

DISCUSSION

Selective precipitation of receptor from cytosol with ammonium sulphate leads to modest purification accompanied by substantial losses. Other workers have obtained similar results in the purification of a calf uterine receptor [4, 6, 24] after incorporating the use of EDTA and calcium ions to produce a receptor form stable against aggregation. The use of the salt precipitation technique with human myometrial cytosol by Notides *et al.* [10] resulted in a 3-fold increase in purity and almost complete removal of serum albumin which is a major contaminating protein [4, 10].

When uterine supernatant from calf was passed through Sephadex G-200 large quantities of estradiol-

Table 3. Purification of estradiol-binding proteins by affinity chromatography on estrone 17-(O-carboxymethyl)oxime-poly(L-lysine)-agarose. The experiments summarized here were performed as described in the methods section. Receptor-[^{3}H]-estradiol complex (R-*E_2) quantities refer to a S.A. of [^{3}H]-estradiol of 46 Ci/mmol

Sample	Volume ml	Total R-*E_2 d.p.m.	R-*E_2/ml d.p.m./ml	Protein/ml mg/ml	Specific activity d.p.m./mg	Recovery %	Purity fold
Cytosol	150	$2{\cdot}74 \times 10^6$	18,000	8·3	2200		
$(NH_4)_2SO_4$ pellet	45	$1{\cdot}41 \times 10^6$	31,000	8·1	3900	51	1·8
Ex affinity adsorbent	25	$0{\cdot}74 \times 10^6$	29,600	$8{\cdot}3 \times 10^{-3}$	$3{\cdot}5 \times 10^6$	27	1600

binding proteins were eluted in highly aggregated state near the exclusion volume [35]. Two more binding components, which were characterized by density gradient analysis as 8S and 4S forms appeared in turn after further elution [35]. In this laboratory no 8S form was encountered on Sephadex G-200 gelfiltration of human myometrial cytosol and aggregated material, if evident at all, occurred only to a minor extent. This result fits in with density gradient analysis evidence which shows that the 7–8S binding protein occurs infrequently and is prone to denaturation on storage. After agarose gel chromatography of human myometrial cytosol, Notides *et al.* [10] reported the appearance of only one estradiol-binding protein having a sedimentation coefficient of 3·3S. The same group also noted the lability of the 8S receptor protein and observed it only in the presence of the proteolytic enzyme inhibitor diisopropylfluorophosphate [10]. Our use of phenylmethyl sulphonylfluoride, which is also a powerful inhibitor of protease activity, however did not have a stabilizing effect on this larger receptor form.

Sedimentation analysis indicates the approximate molecular weights for the 7S and 3·5S receptors to be 160,000 and 45,000 respectively. The result for the smaller binding protein compares favourably with the figure of 38,000 given by Notides *et al.* [10] as the molecular weight of their 3·3S receptor. However, calf uterine cytosol has been shown to contain larger receptor forms—8·6S and 4·5S having molecular weights of 238,000 and 61,000 respectively [6].

The receptor-[^{3}H]-estradiol complex has been shown to be adsorbed by hydroxyapatite. This confirms an earlier observation by Erdos *et al.* [25] who

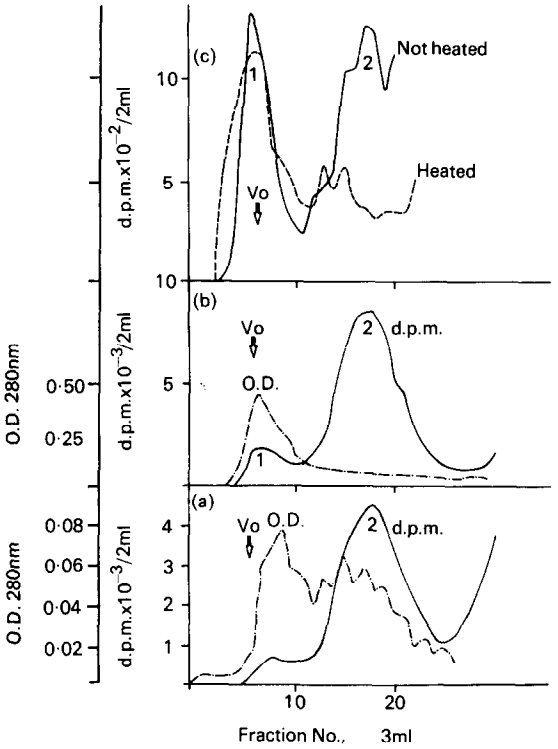

Fig. 12. Sephadex G-200 filtration of affinity chromatography related samples. Aliquots (5 ml) of original cytosol (A), the dissolved pellet from ammonium sulphate precipitation (B) and the sample eluted from the estrone carboxymethyloxime-agarose adsorbent (C) were filtered through Sephadex G-200 (bed vol. 140 ml) packed in $T_{10}K_{200}E_1D_1$, pH 7·4 buffer. Another 5 ml aliquot of the latter sample was denatured by heating at 60°C for 15 min and chromatographed in the same way.

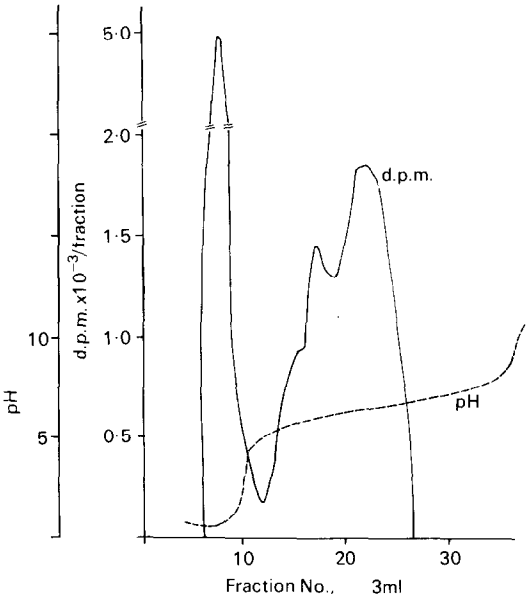

Fig. 13. Electrofocusing pattern of material recovered from affinity chromatography. An aliquot (5 ml) of the sample eluted from the estrone-oxime-agarose derivative was filtered through Sephadex G-50 (bed volume 50 ml) equilibrated in 1 mmol KH_2PO_4 buffer, pH 7·4. The fractions containing macromolecular bound radioactivity were combined and electrofocused in a pH 5–8 gradient over 20 h at 4°C and 400 V. Fractions (3 ml) were measured for radioactivity and pH.

employed this property to separate receptor-bound [^{3}H]-estradiol from the free hormone in a receptor assay method. A separation from some proteins is achieved, but albumin still remains a contaminant. The influence of KCl on the elution of the receptor from hydroxyapatite was not investigated and a KCl instead of a KH_2PO_4 gradient might produce better separation results.

At least two specific estradiol-binding species can be separated by DEAE-cellulose chromatography and both are eluted at low KCl concentration. The levels of these proteins in human myometrial cytosol are storage dependent and there is some evidence supporting a precursor relationship of one to the other. It is not known however if this alteration is reversible. Sedimentation analysis of cytosols containing mixtures of both components indicate that they exist as 4S entities. Chromatography of calf receptor preparations on DEAE-cellulose in other laboratories [4, 35] has shown two estradiol-binding proteins eluting at higher KCl molarities but these were 4S and 8S species. With human myometrial cytosol we have occasionally observed receptor activity on elution with similar KCl concentrations from ion-exchange columns. This could be explained by the presence of an aggregated receptor. Elution of two distinct binding proteins from DEAE-cellulose, both of which sediment at 4S, has been reported for progesterone receptors in chick oviduct [36].

Binding to DNA-cellulose of a receptor-[^{3}H]-estradiol complex prepared from human myometrium has been achieved. Similar binding of steroid–receptor complexes to DNA-cellulose was first established by Mainwaring and Mangan [37] and later confirmed by other investigators [38, 39]. The interaction has been shown by us to be most favoured in low ionic strength media. Toft reported [40] that binding to DNA was facilitated by increasing the KCl concentration to 0·1 M, but found however that the stimulatory effect was not apparent with partially purified receptor preparations [40] and in a more recent article supports our observations [41].

Successful binding to DNA-cellulose has been shown to require a specific receptor conformation which appears to be prevalent in fresh cytosol. Storage of cytosol, either in the frozen state or over extended periods at 4 C precludes DNA binding. Receptor, partially purified from stored cytosols by ammonium sulphate precipitation does not possess increased binding capability. In contrast, Toft noted enhanced DNA binding after similar purification of the estradiol receptor from calf uterus [41]. Other workers [39] have acknowledged the possibility that heterogeneity of the receptor could account for incomplete binding. Varying levels of DNA interaction might result from the influence of factors existing in the cytosol, which are capable of activating the receptor to a suitable form for binding [39]. The efficiency of activation may differ between cytosols. On the other hand it may be that for binding to occur the labile 7–8S receptor must be present. Other workers, however, have shown that the interaction does occur with the 4S form [39]. In studies with the chick oviduct progesterone receptors it has been found that one of the binding components is preferentially adsorbed to DNA while the other binds to purified oviduct chromatin [36]. Puca *et al.* [42] maintain that native DNA bound to cellulose or denatured DNA bound to agarose do not adsorb estradiol receptor complexes from calf uterus. They observe significant binding only after pretreatment of the adsorbents with lysozyme or histones and conclude that this is due to the nonspecific aggregation of the estrogen receptors with these proteins [42].

Although further studies are necessary in order to understand the basis of the interaction between the receptors and DNA it appears that the ability to bind to DNA may provide a criterion for receptor activity and characterization. At the present time *in vitro* binding of estrogen receptors from human myometrial cytosol to purified chromatin as well as their uptake by nuclei, is being studied in this laboratory.

Failure of the receptor to be retained by Con A-Sepharose indicates that the protein is not highly endowed with terminal nonreducing α-D-glucopyranosyl, α-D-mannopyranosyl or β-D-fructofuranosyl residues. Further insight into receptor structure might come from the use of other lectins. Con A-Sepharose chromatography partially purifies the receptor and provides a ready method for removal of a major interfering protein, steroid binding β-globulin.

Indications are that the estrone carboxymethyloxime derivative may be useful in receptor purification. Although the stability tests carried out with the adsorbent may not be conclusive, if any breakdown of the derivative is occurring during use the released ligand does not significantly compete for receptor binding sites. With regard to the purification of the receptor the performance of the affinity adsorbent compares favourably with results achieved by Sica *et al.* [24] and Truong *et al.* [34]. However, certain refinements will have to be introduced before a purification scheme can be formulated. These include an alternative method to ammonium sulphate precipitation for the removal of albumin from myometrial cytosol. Quantitative removal of albumin from plasma by the use of Sepharose-Blue Dextran conjugate has been reported [43] and could be applied to receptor purification. It is also desirable to remove steroid binding β-globulin which could interfere during affinity chromatography. This could be achieved, together with the removal of some other glycoproteins by passage of cytosol through Con A-Sepharose. A suitable purification scheme could then involve filtration of cytosol through Sepharose-Blue Dextran, then through Con A-Sepharose and finally through the estrone carboxymethyloxime-agarose affinity column.

Chromatographic examination of the estrogen receptors present in human myometrial cytosol has

demonstrated that under certain conditions multiple receptor forms exist. Changes in receptor structure or conformation during storage are suggested by differences which have been observed between receptor in fresh cytosol and in cytosol that has been stored either at 4°C or −20°C. Whether these are due to limited proteolysis [10, 44] or to physicochemical changes involving interactions of these macromolecules with other proteins or substances will need further study.

In conclusion the studies reported here indicate the behaviour of the receptor proteins under varying conditions and to different adsorbents. They may assist endeavours to obtain these proteins in homogeneous form.

Acknowledgements—This study was aided by grants from the Saw Medical Research Foundation, the A. Yeldham and M. Raine Medical Research Foundation, University of Western Australia and the National Health and Medical Research Council of Australia.

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