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PURIFICATION AND PROPERTIES OF AN ESTROGEN-BINDING PROTEIN FROM CALF UTERUS*

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SUMMARY

Treatment of uterine cytosol with calcium ions under conditions of high ionic strength to deaggregate the estrogen receptor into sub-units yields a 'stabilized' 4 S binding unit which does not revert to the 8 S form at low ionic strength and which is resistant to aggregation during purification by ammonium sulfate precipitation, gel filtration and ion-exchange chromatography. By this sequence of steps, the calcium-stabilized estradiol–receptor complex from high speed calf uterine cytosol has been purified about 5000 fold and that from low speed cytosol 1000–1500 fold. Further purification of the latter material by disc gel electrophoresis on polyacrylamide gave a product of high purity, showing a single protein band on analytical disc gel electrophoresis and a sharp 4·5 S sedimentation peak of bound estradiol on sucrose gradient centrifugation.

INTRODUCTION AND BACKGROUND

FOLLOWING the demonstration that target tissues for estrogenic hormones possess a unique ability to take up and retain estradiol both *in vivo* and *in vitro*, investigations in a number of laboratories have contributed to our knowledge about the fate of estrogenic steroids in such hormone-dependent tissues as the rat uterus.† Two intracellular sites of estradiol binding are recognized. Most of the uterine hormone is localized in the nucleus from which it can be extracted as a 5 S estradiol–protein complex, but a significant portion (20–30 per cent) is associated with an extranuclear 8 S protein which, at elevated ionic strength, is reversibly deaggregated into a 4 S estradiol-binding sub-unit.‡ From a variety of evidence it appears that the interaction of estradiol with uterine tissue takes place by a two-step sequential mechanism in which the hormone, without chemical alteration, combines with the 4 S binding unit of the 8 S extranuclear protein, activating this moiety to undergo temperature-dependent transformation to the 5 S form accompanied by migration of the estradiol–protein complex to the nucleus. Similar two-step pathways have recently been demonstrated for dihydrotestosterone, proges-

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†Documentation of the many studies of the interaction of estrogens with whole tissues and with broken cell systems is given in references [1–6].

‡The sedimentation coefficient figures of 8, 5 and 4 are convenient approximations based on the position of the sedimentation peaks of crude mixtures in relation to yeast alcohol dehydrogenase, γ-globulin and bovine plasma albumin. The sedimentation rate of the cytosol complex in low salt is somewhat variable depending on the degree of dilution; in high salt, the complex from calf uterine cytosol migrates somewhat faster than the corresponding complex from rat uterus. The actual value for the nuclear complex may be closer to 6 S than to 5. In any case, precise values for the sedimentation coefficients for these proteins must await determination in the analytical ultracentrifuge using the purified substances.

terone, glucocorticoids and aldosterone, suggesting that the activation of an extranuclear receptor protein to enter the nucleus may represent a general pattern for the action of steroid hormones in their respective target tissues.*

To help elucidate the relation between the estrogen-dependent nuclear incorporation of receptor protein and the stimulation of biosynthetic processes leading to growth, we have undertaken the isolation and purification of the estrophilic proteins of calf uterus in quantities sufficient to permit determination of their composition and structure as well as the nature of the 4 S–5 S transformation which is associated with the nuclear fixation of the hormone.

The most favorable receptor substance for attempted isolation is that of the cytosol, inasmuch as radioactive estradiol, necessary to detect and follow the protein during purification, combines directly with this receptor. Initial attempts to purify the estrophilic component of calf uterine cytosol involved affinity chromatography, using a column of estradiol linked to cellulose by an azobenzyl grouping[7, 8]. As has also been observed by others attempting to use affinity chromatography for receptor purification[9, 10], the estradiol column was found to adsorb the cytosol receptor so strongly that it could not be recovered in an active form. Elution of the bound protein was accomplished with deoxycholic acid to yield a material which, on injection into rabbits, produced antibodies capable of precipitating the nuclear estradiol–receptor complex[8], suggesting, in retrospect, an immunochemical similarity between the estrophilic proteins of cytosol and nucleus and supporting the concept that the nuclear receptor is derived from the cytosol protein.

THE CALCIUM-STABILIZED BINDING UNIT

Attempts to employ standard techniques of protein fractionation for purification of the cytosol receptor were hampered at first by the instability of this protein in the crude supernatant fraction where it tends both to decompose and to form large aggregates during storage, salt precipitation or gel filtration. It was then observed[11] that if calcium ions are added to the cytosol fraction of a uterine homogenate, prepared in the presence of EDTA, the estradiol–receptor complex precipitated by 20% ammonium sulfate consists in part of a 'stabilized' sub-unit which does not revert to the 8 S form at low ionic strength and which can be purified without aggregation by gel filtration and ion-exchange chromatography. This stabilized product sediments at about the same rate as bovine plasma albumin (BPA) in low salt sucrose gradients and slightly slower than BPA in gradients containing 300–400 mM KCl, indicating a sedimentation coefficient close to 4·5 (Fig. 1).

Subsequent study of the stabilization phenomenon revealed that calcium ions, in the concentrations employed, have no apparent effect on the 8 S complex itself; only after this entity is deaggregated to the 4 S sub-unit in the presence of salt does the calcium act to destroy its ability to revert to the 8 S form when salt is removed (Fig. 2). Of various divalent cations tested (Ca, Mg, Mn, Zn, Co), only calcium produced the stabilization effect. To obtain the calcium-stabilized 4 S unit, it is important that the original homogenization be carried out in the presence

*A summary of these studies with other steroid hormones, with detailed reference to reports from the laboratories of Liao, Mainwaring, Baulieu, O'Malley, Sekeris, Munck, Tomkins and Edelman, is given in reference[6].

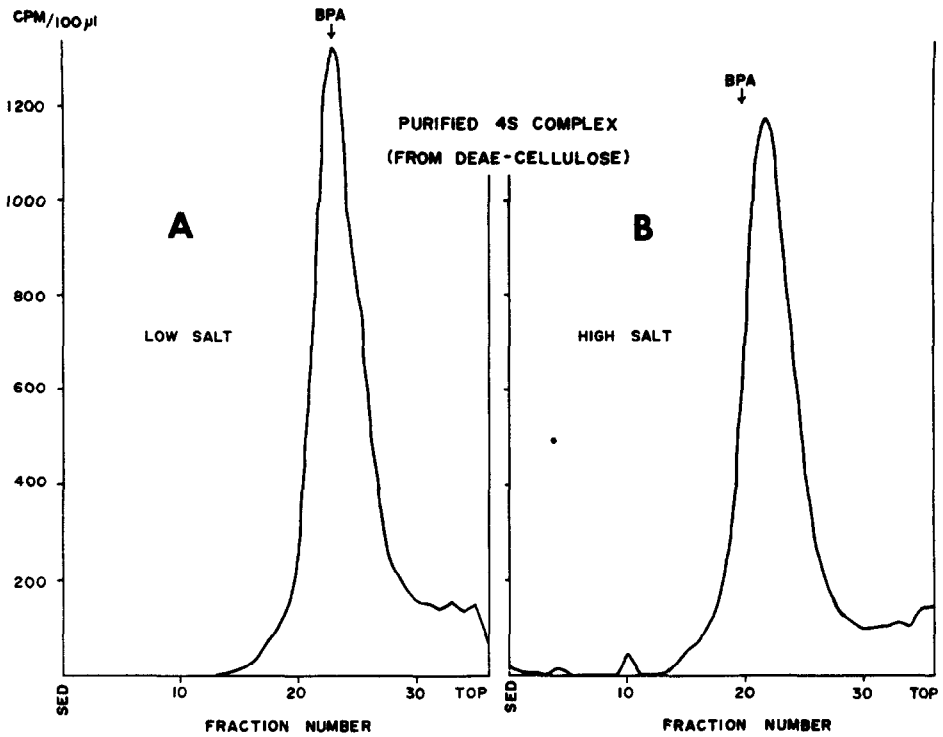

Fig. 1. Sedimentation patterns of purified 4 S complex in (A): low-salt and (B): high-salt sucrose gradients. The calcium-stabilized 4 S complex obtained from low speed calf uterine cytosol and 6,7-^{3}H estradiol (E-2*, S.A. 5·7 Ci/mmole) was purified 1200 fold by ammonium sulfate precipitation, filtration through Sephadex G-200 and chromatography on DEAE-cellulose[11]. Aliquots (0·2 ml) of the solution of purified complex (containing $1{\cdot}25 \times 10^6$ d.p.m./mg protein) were layered on 3·6 ml preformed linear gradients of (A): low salt = 10–30% sucrose in 10 mM Tris buffer, pH 7·4, containing 10 mM KCl and 1 mM EDTA, ($T_{10}K_{10}E_1$) or (B): high salt = 5–20% sucrose in 10 mM Tris buffer, pH 7·4, containing 400 mM KCl, and 1 mM EDTA, ($T_{10}K_{400}E_1$) and centrifuged at 1°C for 12 hr at (A): 317,000 × g or (B): 300,000 × g. Successive 100 μl fractions were displaced by paraffin oil through a bottom puncture into counting vials, and tritium determined by scintillation counting in toluene–Triton X-100 fluor. BPA markers are from concurrent sedimentation of 0·5% solutions of bovine plasma albumin detected by optical density at 280 nm.

of EDTA; addition of EDTA to a cytosol from a homogenate in Tris alone does not seem to substitute for its presence during homogenization.

The foregoing observations indicated that the effect of calcium observed in the purification experiments depends on the ammonium sulfate acting as a dissociating agent as well as a precipitant and suggested that higher yields of stabilized 4 S complex with less accompanying aggregate might be obtained if calcium ions were first allowed to react with the salt-dissociated complex before ammonium sulfate is added to effect precipitation. Such proved to be the case (Fig. 3). With this modification in the precipitation step, the calcium-stabilized estradiol–receptor complex of calf uterine cytosol, prepared on a modest scale (approx. 250 g of tissue) by high speed centrifugation (> 75,000 × g), was purified about 5000 fold by a combination of ammonium sulfate precipitation, gel filtration through Sephadex G-200 and chromatography on DEAE-cellulose[11]. The

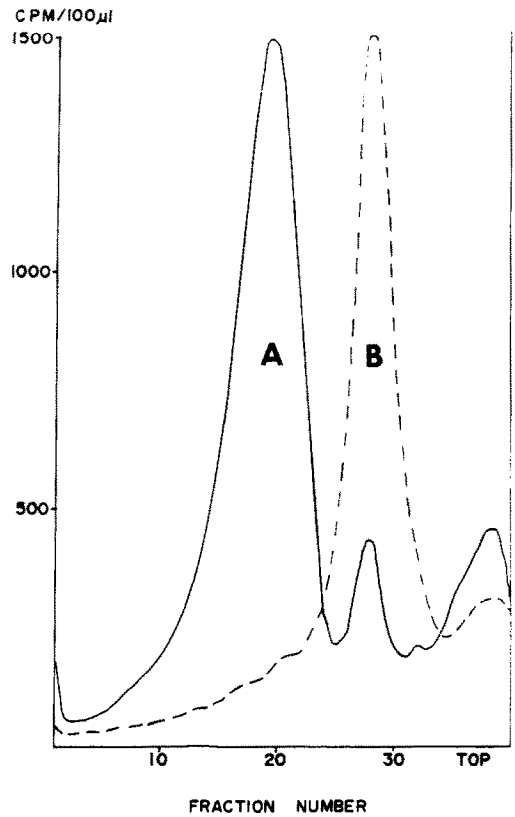

Fig. 2. Irreversible deaggregation of the cytosol complex. High speed cytosol (1·5 hr at 78,000 × *g*), prepared from a homogenate of calf uteri in 4 vol of $T_{10}E_{1·5}$, pH 7·4, was diluted with ¼ vol. of homogenization buffer containing (A): E-2* (57 Ci/mmole) to yield a final concentration of 3 nM or (B): $CaCl_2$, KCl and E-2* to give final concentrations of 4 mM, 500 mM and 3 nM, respectively. After incubation for 3 hr at 2°C, sedimentation analysis of 0·2 ml aliquots was carried out in low salt sucrose gradients, as described in Fig. 1, with centrifugation at 1°C for 12 hr at 308,000 × *g*.

molecular weight of the complex, based on its elution from G-200, appears to be approximately 75,000, and its isoelectric point, as determined by isoelectric focusing, is 6·4[11]. If one estradiol molecule is bound to each receptor unit, the ratio of steroid to total protein indicates that this product is about 4% pure.

Because the cytosol receptor protein is more stable when it is associated with estradiol, the foregoing purification steps were carried out with the hormone–receptor complex. To provide the uncomplexed 4 S unit for evaluation of its ability to associate with estradiol, rapid purification was effected in the absence of estradiol by precipitation with ammonium sulfate and calcium followed by filtration through a short column of DEAE-cellulose under conditions such that the 8 S protein and aggregate are adsorbed but the stabilized 4 S unit is eluted (see legend, Fig. 4). The naked receptor protein, purified in this way by about 150 fold, was found to react with estradiol to form a complex which sediments at about 4·5 S in the absence of salt and which, by the Sephadex G-25 technique of Puca and Bresciani[12], exhibits strong saturable estradiol binding, similar in all respects to the 8 S receptor of the original cytosol (Fig. 4). Both of these

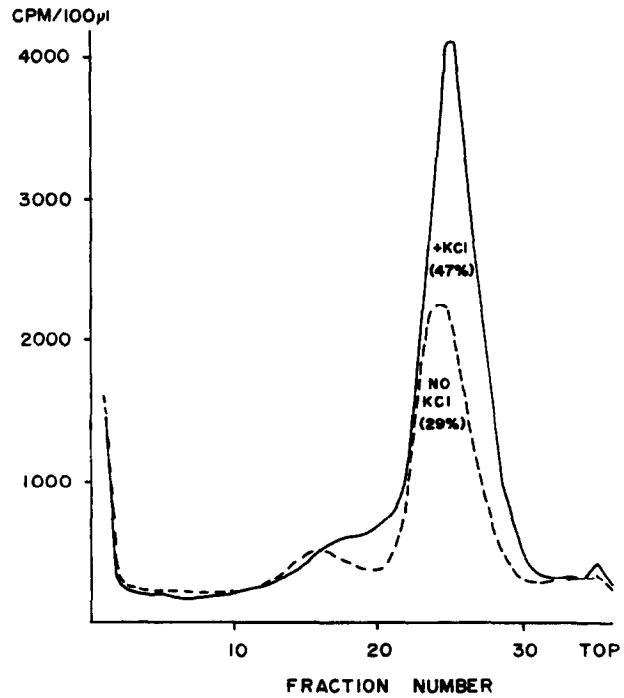

Fig. 3. Ammonium sulfate precipitation with and without predissociation by KCl. High speed cytosol from calf uteri, homogenized at 2°C in 4 vol. of $T_{10}E_{1.5}$, pH 7·4, was made 20 nM in E-2* (5·7 Ci/mmole) and 4 mM in $CaCl_2$ and divided into two parts, one of which was made 1 M in KCl. After standing at 2° for 1 hr, a cold saturated solution of ammonium sulfate (pH 7·2) was added slowly with stirring to each mixture until 20% saturation was reached, after which stirring was continued for one hour in the cold. The precipitates were collected by centrifugation for 20 min at 9000 × *g* and redissolved in one-tenth the original volume of 100 mM Tris buffer, pH 7·4, containing 400 mM KCl and 1 mM $CaCl_2$ ($T_{100}K_{400}C$). After clarification by centrifugation for one hour at 105,000 × *g*, a 0·2 ml aliquot of each mixture was analyzed by sedimentation in a low-salt sucrose gradient for 12 hr at 300,000 × *g* as described in Fig. 1. The percent yield given represents the fraction of the total specific binding capacity of the original cytosol which was recovered as 4 S complex.

binding entities associate more strongly with estradiol than with estrone or estriol, and neither progesterone nor testosterone competes with estradiol for the binding sites. Thus, the stabilized 4 S unit seems to represent the estrophilic portion of the 8 S protein in the cytosol.

LARGE SCALE PURIFICATION

When the foregoing purification sequence was carried out on a larger scale (0·5–1·5 kg uterine tissue), it was necessary to use cytosol prepared by low speed centrifugation (< 10,000 × *g*) because of the limited volume capacity of high speed rotors. With this 'microsome-containing' cytosol, the purification procedure was found to be somewhat less efficient than with high speed cytosol (Table 1). A smaller part of the total cytosol receptor precipitates with 20 per cent ammonium sulfate, and that which does precipitate is accompanied by a greater amount of contaminating material not removed by subsequent gel filtration (Fig. 5). From the elution profiles obtained with the seven liter G-200 column, it is evident that, with

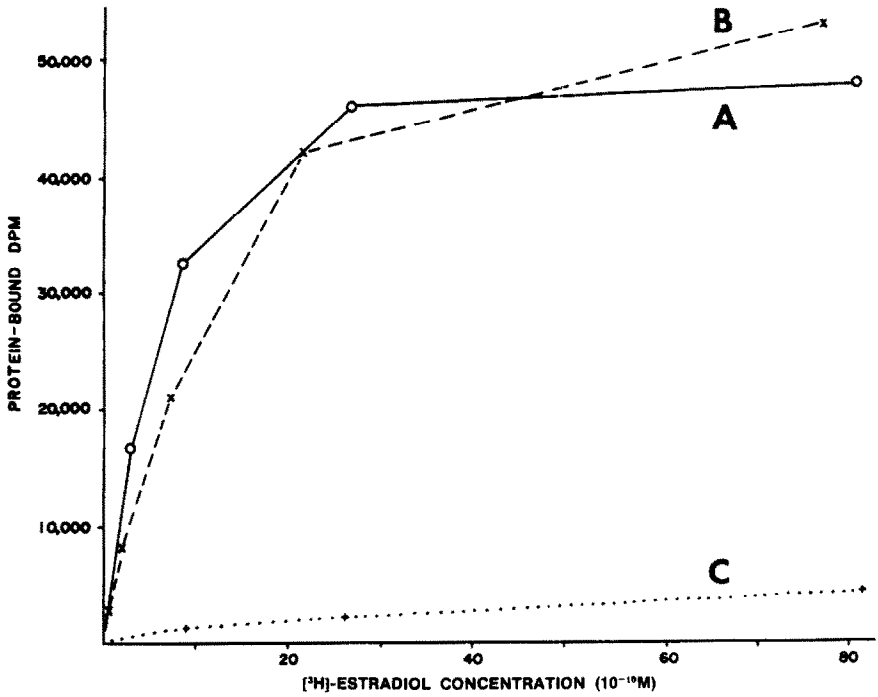

Fig. 4. Association of estradiol with cytosol and with the partially purified, stabilized 4 S receptor protein. The calcium-stabilized 4 S unit was prepared in the absence of estradiol from high speed calf uterine cytosol containing 4 mM $CaCl_2$ by precipitation with ammonium sulfate (20% saturation), redissolving in one-tenth the original volume of $T_{100}C_1$, pH 7·4, and filtering 10 ml of this reconstituted, clarified fraction through a 30 ml column of DEAE-cellulose prepared in $T_{10}K_{10}$ buffer, pH 7·4, under which conditions the stabilized 4 S unit elutes with the unadsorbed protein. Aliquots (0·2 ml) of this 150 fold-purified fraction (A) and the original cytosol (B) were each diluted to 2·0 ml with $T_{10}K_{10}E_1$ buffer, pH 7·4, containing increasing concentrations of E-2* (57 Ci/mmole). After incubation for 1 hr at 2°C, a 1·0 ml portion of each sample (containing 20 μg protein for A and 680 μg protein for B) was analyzed for bound estradiol by filtration through Sephadex G-25 according to the method of Puca and Bresciani[12]. Curve C illustrates similar analysis of the purified 4 S fraction after treatment with E-2* in the presence of a 10 fold excess of unlabelled hexestrol to compete for the sites of specific binding.

Table 1. Typical purification achieved with low speed and high speed cytosol

Stages	Purification factor Low speed	High speed
Supernatant fraction	4	5
Ammonium sulfate ppt[a]	10	12
G-200 eluate	8	20
DEAE-cellulose eluate	4	4
Overall purification[b]	1280	4800

[a]Redissolved in 0·1 the original cytosol volume.
[b]Based on total solids in original homogenate.

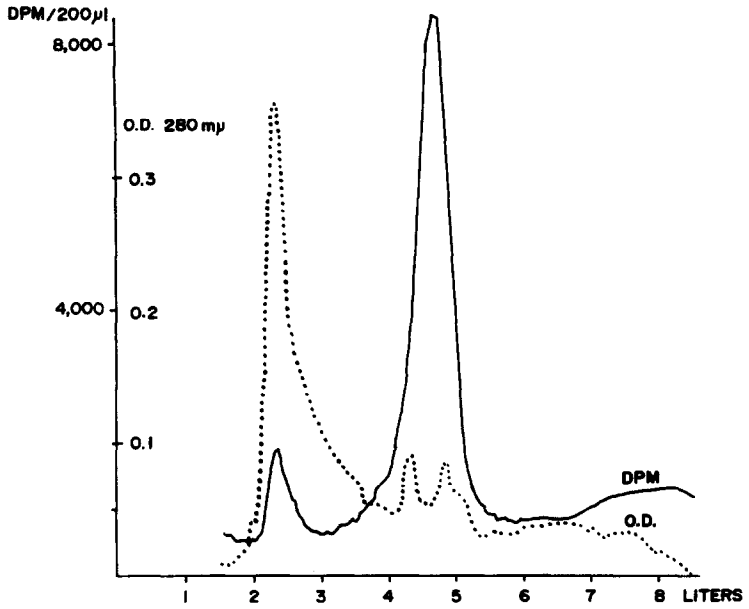

Fig. 5. Purification by Sephadex G-200 filtration of the stabilized 4 S complex from the ammonium sulfate precipitate. Low speed calf uterine cytosol (2650 ml) from a 20% homogenate (w/v) in $T_{10}E_{1.5}$, pH 7·4, was made 15 nM in E-2*, 4 mM in $CaCl_2$ and 1 M in KCl, and, after incubation for 1 hr at 2°C, brought to 20% saturation with ammonium sulfate. After precipitation, redissolving in 265 ml $T_{100}K_{400}C$ and clarification as described in Fig. 3, a 240 ml portion was placed on a 7 liter column of Sephadex G-200, prepared in $T_{10}K_{50}E_1$, pH 7·4, and eluted with the same buffer at a rate of about 100 ml/hr.

low speed cytosol as with high speed, the technique of precipitation with ammonium sulfate after exposure to KCl and calcium favors the production of the desired 4 S complex and minimizes the amount of aggregate and 8 S complex obtained.

The stable 4 S complex can be purified further by ion-exchange chromatography on DEAE-cellulose (Fig. 6). After adsorption of the receptor complex on DEAE-cellulose at low ionic strength, the 4 S binding unit is eluted by a linear KCl gradient, emerging from the column at about 100 mM KCl along with the first protein peak. The major part of the contaminating protein elutes somewhat later, between 150 and 250 mM KCl, with little bound estradiol. The purification achieved by DEAE-cellulose chromatography is usually about 4 fold, but the recovery of complex tends to be low due to some apparently irreversible adsorption on the column.

Assuming one estradiol bound per 4 S unit, the complex obtained at this stage from low speed cytosol is usually about one percent pure. Repetition of either the Sephadex G-200 or the DEAE-cellulose step does not seem to provide any significant further purification; the tritiated estradiol and detectable protein elute together, even when a more gradual salt gradient is used with the DEAE-cellulose column. It would appear that the contaminants remaining after the initial purification by gel-filtration and ion-exchange chromatography are rather similar to the desired receptor in both molecular size and charge, although, as described in the next section, they can be separated further by disc gel electrophoresis.

Because it permits a higher degree of purification to be achieved at this stage,

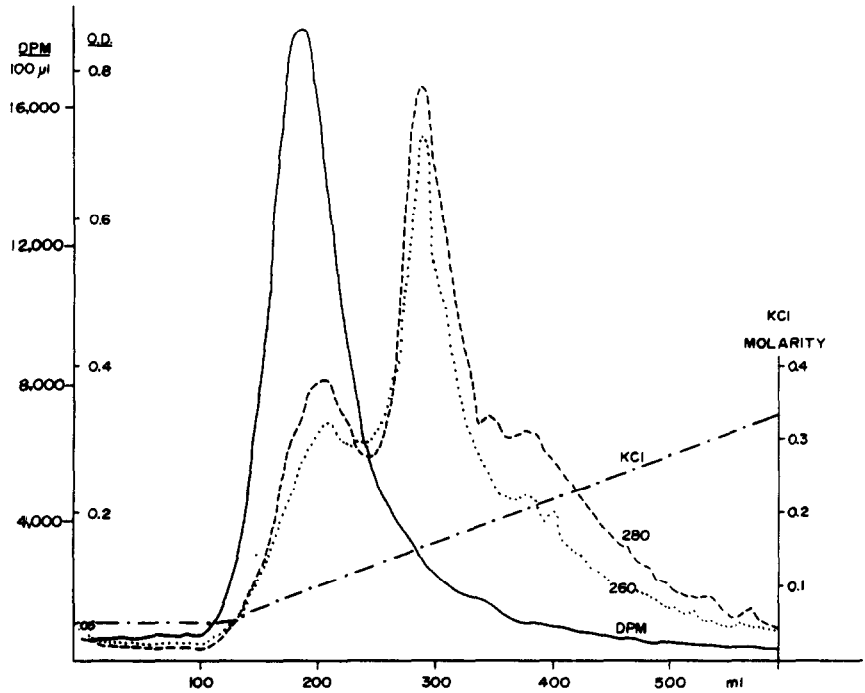

Fig. 6. Purification of the G-200 eluate by DEAE cellulose chromatography. The pooled 4 S fraction (1840 ml containing about 180 mg protein) from Sephadex G-200 purification of the reconstituted ammonium sulfate precipitate of low speed calf uterine cytosol, was applied at the rate of 160 ml/hr to a column of DEAE-cellulose (120 g of Whatman DE 52) prepared in $T_{10}K_{50}E_1$, pH 7·4. After washing with 200 ml of the same buffer, elution was effected by a linear 600 ml gradient of 50 mM to 400 mM KCl in $T_{10}E_1$, pH 7·4. Collection of the fractions illustrated commenced when the salt gradient was applied to the top of the column; KCl concentration in the effluent was measured conductimetrically. Optical density was determined at both 260 and 280 nm.

as well as a greater recovery of product especially in the ammonium sulfate precipitation step, high speed cytosol offers considerable advantage over low speed cytosol as starting material. For that reason, we have recently installed equipment for the large scale preparation of high speed cytosol, which will be used in future purification experiments.

ELECTROPHORETIC PURIFICATION

Although isoelectric focusing provides a convenient analytical method for determining the isoelectric point of the partially purified estradiol–receptor complex[11], it did not prove useful for further purification because of the decomposition of the receptor which takes place at its isoelectric point (6·4) during the 12–16 hr required for resolution. It was found that disc gel electrophoresis on polyacrylamide, which is complete within a few hours at pH 9 where the complex is more stable, is highly effective for the further refinement of the partially purified complex. As illustrated in Fig. 7 for the successive purification steps with low speed cytosol, the major contaminants remaining after DEAE-cellulose chromatography are indicated by three intense bands migrating about one-half as rapidly as albumin (the large fast band observed at the earlier stages

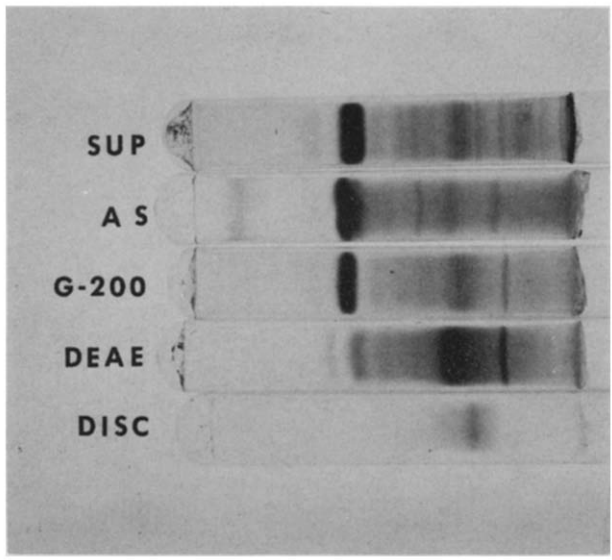

Fig. 7. Acrylamide disc gel electrophoretic analysis at various stages of purification of calcium-stabilized 4 S complex from low speed cytosol. Samples were run in a Canalco apparatus with ice bath cooling using 7% acrylamide gels in Tris-glycine buffer at pH 9 and a current of 2·5 mA per tube. The electrophoresis was terminated when the tracking dye reached the bottom of the separating gel. For each sample except DISC, 0·2 ml of sample gel was prepared to contain 100–200 μg of protein. The G-200 and DEAE-cellulose purified samples were concentrated prior to electrophoretic analysis, using Diaflo XM-50 membranes. For the DISC sample, comparable 1·6 mm slices from 5 analytical gel columns, run in parallel using the DEAE-cellulose purified receptor as sample, were combined and extracted with $T_{10}K_{50}E_1$ buffer, pH 7·4; 0·5 ml of extract from the slices containing the peak concentration of tritiated estradiol was used to prepare a 1·0 ml sample gel for the analytical run. After removal from the columns, the separating gels were stained overnight with amido schwarz reagent and then destained electrophoretically.

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of purification and removed by DEAE-cellulose) and a single band running about one-third as fast as albumin. The radioactivity of the estradiol–receptor complex migrates as a discrete area, well ahead of this single band and just behind the slowest component of the triplet.

The radioactive regions from five such analytical gel electrophoresis columns, run in parallel, were combined and extracted with buffer. When the concentrated extract was examined by sucrose gradient ultracentrifugation, the radioactivity was found to sediment sharply at about 4·5 S. When it was again subjected to disc gel electrophoresis, the radioactive extract showed a single protein band by amidoblack stain (DISC – Fig. 7). Inasmuch as this particular product from low speed cytosol had reached 1000 fold purification after DEAE-cellulose chromatography, representing about one percent purity, it would appear from the disappearance of the intense contaminant bands of the DEAE tube that the complex, after refinement by disc gel electrophoresis, is probably of at least 50% purity. If so, the single protein band in the DISC tube represents the first time, at least in our laboratory, that the receptor protein has been detected by any criterion other than the radioactivity of the estradiol bound to it.

Application of preparative scale disc gel electrophoresis to the 5000 fold purified product obtainable from high speed uterine cytosol should yield the 4 S estrogen-binding unit in a form suitable for studies of composition and structure. These experiments are currently in progress in our laboratory.

ACKNOWLEDGEMENTS

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