PURIFICATION OF ESTRADIOL RECEPTOR FROM RAT UTERUS AND BLOCKADE OF ITS ESTROGEN-BINDING FUNCTION BY SPECIFIC ANTIBODY

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Summary -- Protein fractions which selectively bind ^{3}H-estradiol-17β have been isolated from rat uterus by gel filtration and repeated ion-exchange chromatography. The purified preparation of major activity migrates essentially as a single component upon disc gel electrophoresis at pH 8.1 and 4.5, or on immunoelectrophoresis. Anti-receptor γ-globulin selectively abolishes the estrogen-binding peak which characteristically sediments at "9.5 S" on sucrose gradient centrifugation.

The uterus possesses apparently unique proteins which, by virtue of their selective interaction with estrogens, have been termed "estrogen receptors" (1, 2). Substances with these properties are observed in uterine homogenates both in the 105,000g supernatant (3, 4, 5) and in extracts of the nuclear fraction (6). In this paper, we report on the purification of protein receptor from the uterine 105,000g supernatant from intact adult rats. Moreover, we have established by immunological methods that product corresponds to the active portion of the starting material.

Measurement of Binding Activity -- When our preparations (7, 8) of the 105,000g supernatant were subjected to sucrose gradient (5-20%) centrifugation, ^{3}H-estradiol sedimented in association with macromolecules corresponding to the "9.5 S" component described by Toft and Gorski (4, 5)[1]. Estradiol-binding material also was characteristically eluted in the void volume (Vo) fraction of Sephadex G-200 columns[2]

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[1] Our values were approximately 9.1 S, using yeast alcohol dehydrogenase (7.6 S) as standard (9).

[2] Prior to gel filtration, ^{3}H-estradiol was equilibrated in vitro for 1 hr at 2^{o} with either uterine homogenate, 105,000g supernatant, or fractions purified from the latter (7).

Accordingly, for assay purposes, binding activity was expressed as cpm in the Vo relative to the total units of absorbance at 280 mμ (A_{280})[3] of all the material eluted. Saturation of the binding sites was denoted by the presence of unbound hormone in the retarded volume of the columns. The advantage of the present assay over other methods which measure overall binding (i.e. equilibrium dialysis, ultrafiltration), is that a distinction is made between uterine protein and blood contaminants such as albumin, which is eluted at a later stage (7).

Purification Procedure (cf. 7) -- Uteri from rats 3-5 months old were frozen in liquid nitrogen and pulverized in stainless steel percussion mortars. All subsequent operations were carried out at 0-4°. The pulverized material was homogenized in 0.054 M sodium phosphate buffer containing 0.005 M mercaptoethanol, pH 7.7. The ensuing 15-20% homogenate was centrifuged at 105,000g for 75 min. Supernatant from 35 (or fewer) uteri was applied to a column of DEAE-Sephadex A-50 (1.5 x 28.0 cm) previously equilibrated with homogenization buffer. The column was then washed with 0.1 M NaCl in buffer. The estradiol-binding fraction was eluted with 200 ml of 0.3 M NaCl in phosphate buffer and dialyzed against 4 volumes of saturated $(NH_4)_2SO_4$, pH 7.7.[4] The resultant precipitate was suspended in 5 ml, and dialyzed against, starting buffer. The DEAE-Sephadex step provided an approximately 5-fold enrichment of the receptor fraction on the basis of removal of extraneous protein. The solubilized material was then applied to a Sephadex G-200 column (2.5 x 90.0 cm) and eluted with phosphate buffer at a flow rate of about 20 ml/hr.[2] Protein associated with ^{3}H-estradiol eluting in the Vo represented an additional enrichment of about 3-fold. This fraction was then reapplied to a DEAE-Sephadex column which had been equilibrated with initial phosphate buffer. Gradient elution was carried out as indicated in Fig. 1. The pattern obtained was highly reproducible when the material chromatographed was within the range of 25-125 A_{280} units.

Results and Discussion -- Fractions within each peak (B-F) were pooled as indicated in Fig. 1, concentrated, dialyzed and assayed. These five preparations all bound

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[3] A_{280} throughout refers to quantity, not concentration.

[4] Alternatively, concentration was achieved in a Diaflo ultrafiltration apparatus with an XM-50 membrane (Amicon Corp., Cambridge, Mass.)

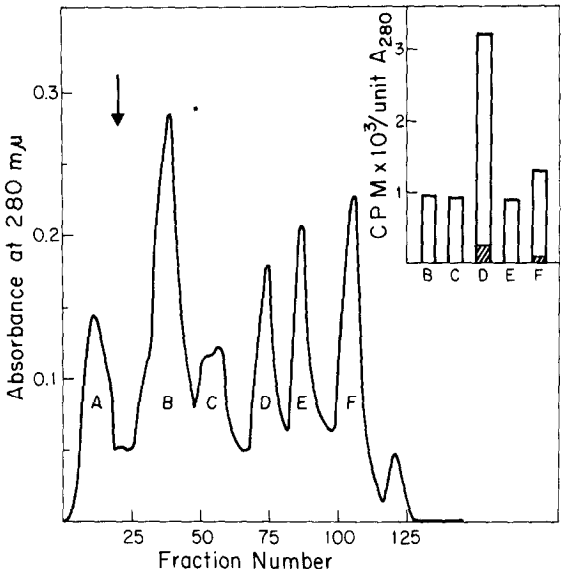

Fig. 1 -- Linear gradient elution profile from DEAE-Sephadex. The Vo fraction from the preceding step (preparative Sephadex G-200, approximately 125 A_{280} units, originating from the 105,000g supernatant from 70 rat uteri), was applied to the column (1.5 x 28.0 cm). Elution was carried out with 1 liter of a gradient, starting at arrow, between 0.1 and 0.4 M NaCl in phosphate buffer. Fractions of 8 ml were taken, pooled as indicated (B-F), and concentrated. Following dialysis, the estradiol-binding activity was assayed (cf. text and ref. 7), with results shown in the insert graph. Height of bars represents estradiol-binding activity. Where indicated (cross-hatching), the binding of ^{3}H-testosterone, normalized to the specific activity of estradiol, was determined.

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^{3}H-estradiol to some degree, but the greatest relative binding by far was to sample D[5]. Disc gel electrophoresis of this material, obtained by gradient elution from DEAE-Sephadex when 25 A_{280} units were chromatographed, gave a single band staining with amido black (Fig. 2). In contrast, the other chromatographic peaks were relatively heterogeneous (7). If more than 25 A_{280} units were applied to the column under the conditions described, the resultant disc electropherograms of fraction D were proportionately contaminated.

Specificity of the purified fraction for estradiol binding was indicated by its low degree of association with ^{3}H-testosterone (Insert, Fig. 1). The ratio of estradiol to testosterone bound by either fraction D or F was about 14. The slight extent of inter-

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[5] The yield of this product was approximately 250-500 μg from 35 uteri (ca. 12 g).

action of testosterone with the purified fractions (Fig. 1), and with the 105,000g supernatant[6] (data not shown), may form the molecular basis for the uterotrophic effect of the androgen (12, 13).

The binding activity of fraction D was 6-9 times greater than that of the starting material (7). This seemingly modest degree of purification is inconsistent with the

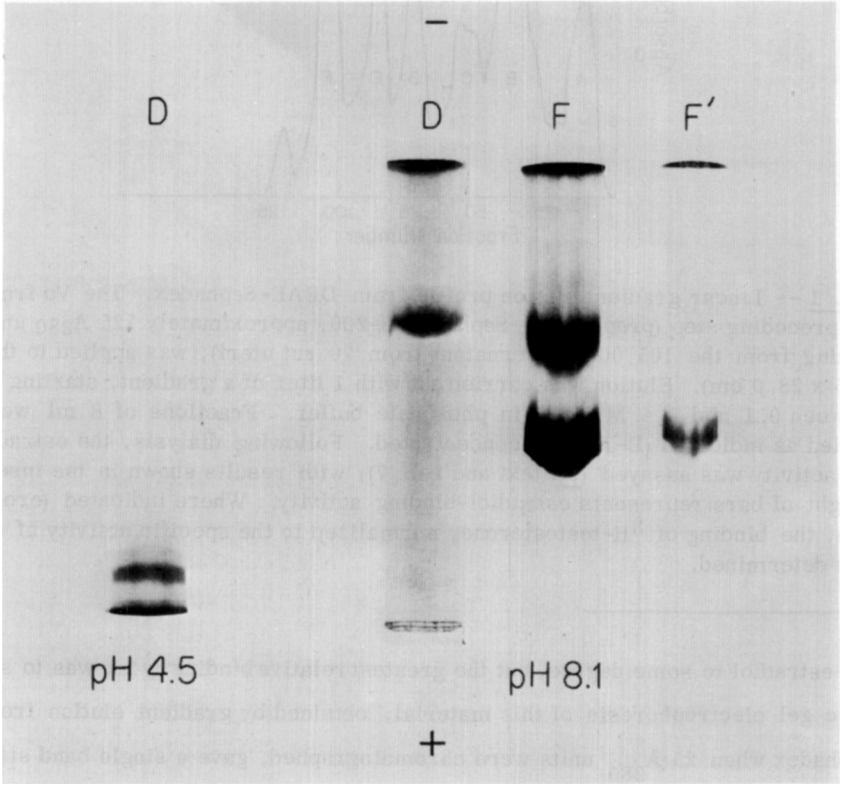

Fig. 2 -- Disc gel electropherograms of DEAE-Sephadex fractions D, F and F' originating from various runs. Electrophoresis in 5% polyacrylamide gels was conducted either with pH 8.1 or pH 4.5 electrolyte systems according to Clarke (10) or Reisfeld et al. (11), respectively, with the exception that urea (8 M sample solution, 5 M gel) was utilized. Fraction F' was purified in the same manner as described in the text for fraction F except that a narrower gradient (0.15-0.40 M NaCl in phosphate buffer) was employed for elution from DEAE-Sephadex. It will be noted that F' was thus rendered free of contaminants corresponding to fraction D.

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[6] Interaction of testosterone with the 105,000g supernatant was too low to be detectable by sucrose gradient analysis, in contrast to the results with gel filtration.

fact that the bulk of apparently extraneous protein is discarded as the result of isolation. Therefore, it seemed mandatory to relate the purified product to the estradiol-binding protein as it occurred in the starting material to ascertain whether or not a contaminant had been purified inadvertently. This end was achieved with unequivocal results through the use of specific rabbit antibody preparations. Thus, the addition to uterine 105,000g supernatant of γ-globulin[7] directed against either fraction D or F' (cf. Fig. 2) virtually eliminated the 9.5 S ^{3}H-estradiol peak when the mixtures were subjected to sucrose gradient centrifugation (Fig. 3). The loss in receptor activity

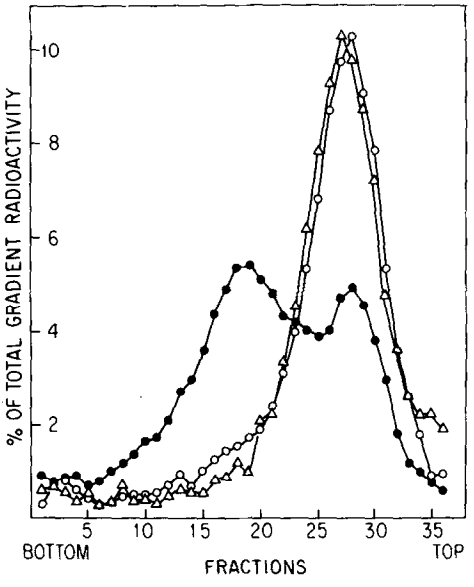

Fig. 3 -- Elimination of 9.5 S receptor binding activity with anti-D and anti-F' γ-globulins. Sucrose gradient profiles of 105,000g supernatant in the presence of anti-D (Δ-Δ), anti-F' (o-o), or absorbed anti-F' (•-•) preparations are shown. A near-optimal proportion of antibody was incubated with fresh 105,000g supernatant for 2 hr at 2°. After centrifugation to remove precipitate, the mixture was equilibrated with 5.0×10^{-5} μg/ml ^{3}H-estradiol (40.0 C/mmole) for 1 hr at 2°. A 0.2 ml aliquot (containing ca. 1,300 cpm) was layered onto a 4.6 ml linear gradient of 5-20% sucrose in phosphate buffer and centrifuged for 7 hr at 50,000 rpm in a Spinco SW-50 rotor. Thirty-six equal fractions were collected directly into liquid scintillation counting vials. Radioactivity was determined (cf. 7) with an efficiency of about 35%.

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[7] Antiserum (14) was purified by two successive precipitations at 1/3 saturation with ammonium sulfate, pH 7.8, to remove rabbit serum albumin which binds tritiated estradiol and thus obscures the sedimentation profile.

was accompanied by a commensurate increase in estradiol binding to a component corresponding to serum albumin (7). Antibody to F' which had been absorbed previously with a Vo preparation from 105,000g supernatant failed to eliminate 9.5 S-binding (Fig. 3). Similar results were obtained with absorbed anti-D, and with γ-globulin preparations of non-immune rabbit serum (data not shown). Not unexpectedly, both anti-D and anti-F' formed essentially single, identical precipitin arcs upon immunoelectrophoresis of uterine 105,000g supernatant. The fact that the two fractions, distinct with respect to ion-exchange and disc gel electrophoretic properties, are nevertheless identifiable immunochemically as 9.5 S receptor may indicate polymorphism of the native material. On the other hand, available evidence (14) suggests that variable degrees of denaturation (cf. 15) of the receptor molecule are responsible for the wide distribution of binding activity in eluates from DEAE-Sephadex. In harmony with this suggestion is the observation that when fraction D was rechromatographed on DEAE-Sephadex, protein eluted over the entire gradient range and was particularly abundant in a position corresponding to that of fraction F. Denaturation is likewise believed to be the cause of losses in receptor activity occurring during purification, and of its lability in general (7).

The present experiments imply that the minor increase in binding activity of either fraction D or F does not correctly reflect its degree of purification. Regardless of quantitative considerations, however, estradiol receptor of the soluble fraction from uterus has been purified essentially to homogeneity by the criteria of two of the most sensitive and selective analytical methods available -- disc gel electrophoresis and immunoelectrophoresis. Moreover, the present data provide the first evidence for selective blockade of estrogen binding by 9.5 S receptor with specific antibody.

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Erratum

Vol. 33, No. 3 (1968), in the Communication, "The Degradation of Twisted Circular Lambda DNA by Phage Lambda Mutants," by Robert C. Shuster and Arthur Weissbach, pp. 514-519:

The mutant designated in the paper as "λ C_I857 434hy int 4 Sus P_3" should read:

"λ C_I857 434hy int 41 Sus P_3."