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ALPHA-FETOPROTEIN: SECONDARY ESTROGEN BINDER IN IMMATURE RAT UTERINE CYTOSOL

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SUMMARY

Alpha-fetoprotein (AFP), an estrogen(E)-binding glycoprotein in the serum of the immature rat, has been shown to be present in uterine cytosols and to migrate as 4*S* in sucrose density gradients. Evidence is presented here which (1) indicates that AFP is not a component of the 8*S* uterine E-binding complex in hypotonic cytosol (20 day old rat), and (2) confirms AFP as the E-binding moiety observed in the cytosol only at E concentrations which exceed that required to saturate the 8*S* form. Thus, increased E (5–1000 nM) yields increased E binding in the 4*S* rather than 8*S* region. Binding in the 8*S* region is eliminated by elevation of temperature; in contrast, AFP is thermostable. Removal of KCl from hypertonic cytosol containing [^{3}H]-estradiol and [^{125}I]-AFP followed by sucrose density gradient ultracentrifugation of the resulting hypotonic cytosol did not effect incorporation of [^{125}I]-AFP into the 8*S* complex. Estradiol could be definitively associated with AFP in uterine cytosol only after steady-state polyacrylamide gel electrophoretic separation at 50 nM[^{3}H]-estradiol. It is concluded that the thermolabile E-binding component of uterine cytosol which is saturated at 10 nM E and which is observed in the 8*S* region in a hypotonic environment is not and does not contain AFP; rather, AFP is the thermostable, higher capacity, lower affinity E-binding component observed in the 4*S* region.

INTRODUCTION

Uterine cytosol of the immature rat contains a serum estrogen-binding protein which migrates as 4*S* in sucrose density gradients. Binding of estradiol to this component is seen to increase as the estradiol concentration increases beyond the 10 nM concentration required to saturate the 8*S* binding form [1]. The 4*S* component which is labeled at 1 nM and first observed in 5 day old rat uterine cytosol decreases with age while the 8*S* receptor increases so that in 20 day old rats only the 8*S* complex is observed [2]. On the basis of steroid affinity, specificity and immunoadsorption studies the 4*S* component has been identified as alpha-fetoprotein (AFP), a serum α_1-globulin [3, 4]. It has been proposed that AFP in hypotonic cytosol is associated with some other intracellular constituent and is thus an, if not the, estrogen-binding subunit of the higher affinity 8*S* complex [5]. In this configuration, the AFP is presumed to be unavailable for interaction with specific anti-AFP antibodies. However, since the binding properties of the receptor and AFP are so different in high salt medium, others have concluded that they are two distinct and separable proteins [6].

Evidence is presented here to show that while AFP is indeed present in uterine cytosol, it is not part of the 8*S* estrogen-binding component observed at estrogen concentrations at which association with the 8*S* but not the 4*S* is saturated. This paper reports (1) the increased low salt charcoal resistant 4*S* binding or tritiated estradiol (*E) at labeling concentrations up to 1000 nM; (2) the instability of the 8*S* complex at 57°C in contrast to AFP; (3) the lack of incorporation of AFP into the 8*S* complex after removal of salt from high salt cytosol containing [^{125}I]-AFP; and (4) the association of *E with AFP after steady-state polyacrylamide gel electrophoresis of 50 nM-*E-labeled cytosol.

MATERIALS AND METHODS

Animals. Female Sprague–Dawley rats 20 days old were decapitated and exsanguinated. Blood was collected, allowed to clot and centrifuged. Serum was stored at −70°C until used. Uteri were removed, stripped of fat and adherent tissue, rinsed for 15 min at 0–4°C in 10 mM Tris-HCl, pH 7.4 at 20°C, 1.5 mM EDTA (TE buffer) and used immediately for cytosol preparation.

Cytosol. Uteri were homogenized in TE buffer in a glass–glass homogenizer at 0–4°C, the temperature at which all subsequent procedures were performed. The homogenate was centrifuged at 105,000 × *g* for 1 h and the resulting supernatant referred to as TE buffered cytosol. For hypertonic cytosols crystalline KCl was added to make TEK buffered cytosol (0.4 M KCl). The cytosol of one uterus is referred to as a "unit" (U), i.e. 4 uteri homogenized in 1 ml yields 4 U/ml or 1 U/0.25 ml.

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Immunochemicals. Goat anti-rat AFP-serum (anti-AFP), prepared [7] and supplied by Dr S. Sell, University of California, San Diego, and normal goat serum (NGS) were diluted (1:10) with TE buffer, stored at −70°C and used at a final dilution of 1:100.

Radiochemicals. [2,4,6,7-^{3}H]estradiol (*E, 95–102 Ci/mmol) was obtained from New England Nuclear, evaporated to dryness, reconstituted in ethanol and stored at 0–4°C. Rat alpha-fetoprotein (AFP) purified from amniotic fluid [8] and supplied by Dr S. Sell was used for labeling with [^{125}I] to a specific activity of 20 μCi/μg by a modified method of Bolton and Hunter[9]. The radioiodinated reagent (^{125}I-3-[4-hydroxyphenyl]propionic acid *N*-hydroxysuccinimide ester, 1500 Ci/mmol) was obtained from New England Nuclear.

Estrogen binding. TE buffered cytosol was added to glass tubes containing *E and incubated at 0–4°C for 2 h. An equal volume of 0.05% dextran (w/v)–0.5% charcoal (w/v) suspension in TE buffer (DCC) was added and the mixture incubated at 0–4°C for 30 min. After centrifugation (2000 × *g* × 10 min) ^{3}H was measured in an aliquot (0.1 ml) of the supernatant and the remainder used immediately. The cytosol was either incubated at 57°C for 30 min, or maintained at 0–4°C.

Gel filtration. Bio-gel P-10 (Bio-Rad) beads were swollen in TE buffer, packed in glass pipets (5 × 85 mm) and equilibrated in TE buffer containing 0.1% gelatin (w/v, TEG). 500 μl of TEK buffered cytosol (4U/ml) unlabeled or labeled at 5 nM *E, and containing 1.1 ng of [^{125}I]-AFP was applied to the column and eluted in 10 drop (0.25 ml total) fractions with TEG buffer at 25°C; void volume = 1.5 ml.

Sucrose density gradient ultracentrifugation (SDG). Aliquots (0.3 ml) of cytosol or serum (20 day old female) were layered on top of 5–20% linear sucrose gradients (TE or TEK buffer) and centrifuged at 45,000 rev./min for 16.5 h at 2°C using an SW-60 rotor in a Beckman L2-65B ultracentrifuge. ^{14}C-labeled rat serum albumin (Alb, 4.3*S*) was included as standard. AFP-bound *E was analyzed in a separate gradient. Alb and AFP migrated the same distance and are referred to as "4*S*", and the 7–8*S* complex as "8*S*". All gradients were fractionated (0.2 ml/fraction) and counted for radioactivity. The uncounted portions of the three fractions comprising the 4*S* region of the serum were combined for subsequent electrophoresis.

Steady-state polyacrylamide gel electrophoresis (*SPAGE*). Sera from 1 or 20 day old rats were diluted 1:20 with TE buffer containing 5% sucrose (w/v) and incubated with 50 nM *E (0–4°C × 2 h). TE buffered cytosol (10 U/ml) containing 5% sucrose (w/v) was incubated with 10 or 50 nM *E (0–4°C × 2 h). Aliquots (0.05 ml) of all incubations were layered on top of vertical 6.5% polyacrylamide disc gels (5 × 70 mm), pH 8.9, prepared according to Davis[10] containing equimolar *E. *E was incorporated into the gels because conventional electrophoresis had proven unsuccessful. Electrophoresis was performed at 4°C for 70 min at a constant current of 4 mA/gel at steady-state conditions [11]. Protein was stained with 1% amido black while companion gels were sliced (1.5 mm) and the radioactivity determined.

Radioactivity measurement. Samples of cytosol (0.1 ml), gel filtration (0.25 ml) or sucrose gradient fractions (0.05 or 0.2 ml) were counted for ^{3}H and ^{14}C in 4.5 ml of Formula 947 (New England Nuclear). Gel slices were incubated in 0.4 ml Protosol (New England Nuclear)–toluene–water (6:3.5:0.5 by vol.) at 55°C × 5 h [12] prior to counting. Counting efficiencies in a Packard 3320 liquid scintillation spectrometer were 28–32% for ^{3}H, or 18.5% and 70% for ^{3}H and ^{14}C, respectively. Sucrose gradient fractions (0.2 ml) containing ^{125}I were counted at 79% efficiency in a Nuclear Chicago 1185 automatic gamma counting system. Samples containing both ^{125}I and ^{3}H were counted first in a gamma counter and then in a liquid scintillation counter.

RESULTS

Increased 4S estrogen binding with increased estrogen concentrations

The results illustrated in Fig. 1 indicate that increases in the *E concentration in immature rat uterine cytosol (20 day) result in large increases in *E binding to 4*S* rather than 8*S* components. TE buffered cytosol (2 U/ml) was incubated with *E at concentrations of 5, 10, 50, 100, 500 or 1000 nM. After adsorption of free (unbound) or loosely associated

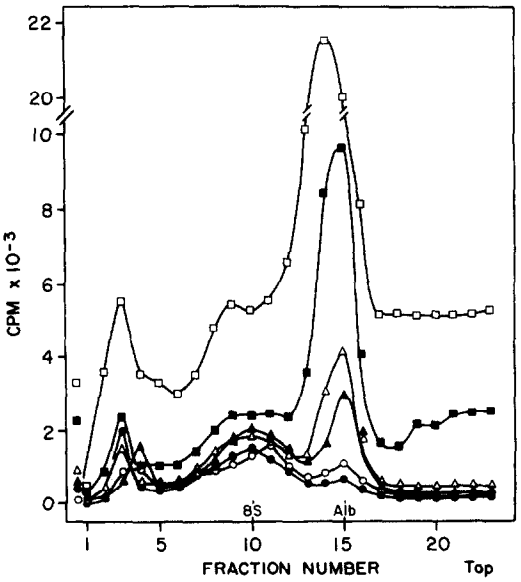

Fig. 1. Increased 4*S* estrogen binding with increased estrogen concentrations. TE buffered cytosol (2 U/ml) was incubated (0–4°C × 2 h) with *E at a concentration of 5 (●——●), 10 (○——○), 50 (▲——▲), 100 (△——△), 500 (■——■) or 1000 (□——□) nM. Unbound *E was adsorbed with DCC (1:1) and 0.3 ml aliquots (0.3 U) were analyzed by SDG. Radioactivity was measured in 0.2 ml fractions. [^{14}C]-albumin (Alb) was used as an internal standard for all gradients (4.3*S*, fraction number 15).

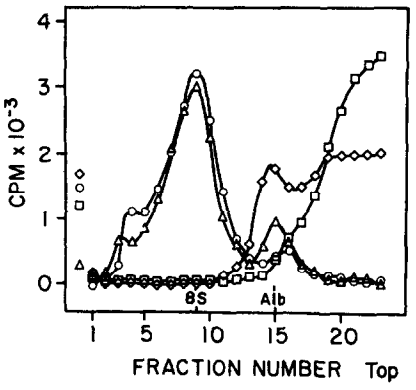

Fig. 2. Loss of 8S estrogen binding after temperature elevation. TE buffered cytosol (1 U/0.15 ml) ± AFP (60 pmol) was incubated (0–4°C × 2 h) with *E (5 nM). Unbound *E was adsorbed with DCC (1:1) and 0.3 ml aliquots (1 U) were either maintained at 0–4°C (—AFP, O——O; +AFP, △——△) or incubated at 57°C (—AFP, □——□; +AFP, ◇——◇) for 30 min. The samples were analyzed by SDG and the radioactivity measured in 0.2 ml fractions.

(albumin) steroid with DCC (1:1), 0.3 ml aliquots (0.3 U) were analyzed by SDG. At 5 nM 0.30 pmol/U of *E were localized in the 8S region (3 peak fractions) and 0.11 pmol/U in the 4S region; at 500 nM *E in the 8S region increased only 0.7-fold (0.51 pmol/U) compared to a 19-fold increase (1.90 pmol/U) in the 4S region. At 1000 nM, 4.24 pmol/U were bound to the more slowly sedimenting 4S form. Note that 3.50 ± 0.25 (mean ± S.E.M.) pmol of immunoassayable AFP per unit of uterine cytosol has been determined for the 20 day rat (Linkie and LaBarbera, unpublished observations).

Loss of 8S estrogen binding after temperature elevation

Heating of TE buffered cytosol for 10 min at 57°C abolished estrogen binding to the 8S component without increased binding in the 4S region (Fig. 2). TE buffered cytosol (6.7 U/ml) ± AFP (400 pmol/ml) was incubated with 5 nM *E, DCC-treated and either heated at 57°C or maintained at 0–4°C for 30 min. Aliquots (0.3 ml, 1 U) were analyzed by SDG. It can be seen that the addition of 57 pmol/U of purified AFP to cytosol effected an increase only in the 4S region. In heated cytosol without exogenous AFP, the released 8S-associated *E was free rather than localized in the 4S region. The 4S *E peak observed in heated cytosol containing AFP—a heat-stable estrogen-binding protein [13]—includes both free steroid and that bound to the added AFP.

Lack of incorporation of [^{125}I]-AFP into the 8S estrogen-binding complex

Figure 3 illustrates that immunoreactive [^{125}I]-AFP did not migrate as a component of an 8S complex in SDG after TEK buffered cytosol containing [^{125}I]-AFP was desalted by gel filtration. TEK buffered cytosol (1 U/0.27 ml) containing

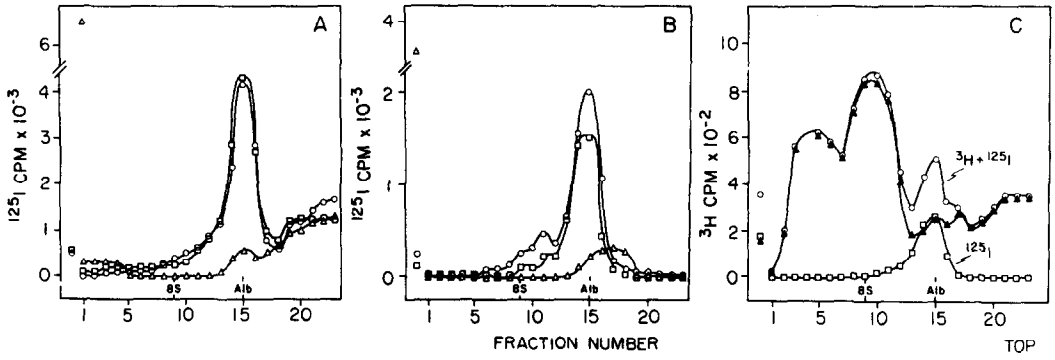

Fig. 3. Lack of incorporation of [^{125}I]-AFP into the 8S estrogen-binding complex. TEK buffered cytosol (1 U/0.27 ml) containing [^{125}I]-AFP (2.2 ng/ml) was incubated with 5 nM *E. The samples (0.27 ml) were incubated with 0.03 ml of either TE buffer (O——O), normal goat serum (NGS, 1:100 final dilution, □——□) or goat anti-AFP serum (1:100 final dilution, △——△) at 0–4°C for 3 h and analyzed by SDG in the presence of 0.4 M KCl. The gradient fractions (0.2 ml) were counted in a gamma spectrometer. The [^{125}I]-AFP profiles are shown in (A). The [^{125}I]-AFP was observed to migrate as 4S in SDG containing 0.4 M KCl in the TE buffer and NGS controls, but was complexed and precipitated only by anti-AFP serum. KCl was removed from duplicate samples (2 U/0.54 ml) by gel filtration, the 4 peak fractions (void volume) of the column eluate pooled, and aliquots (0.27 ml, 0.3 U) incubated with 0.03 ml of either TE buffer (O——O), NGS (□——□) or anti-AFP serum (△——△), analyzed by SDG in the absence of KCl and the gradient fractions counted in a gamma spectrometer. The [^{125}I]-AFP profiles shown in (B) indicate that [^{125}I]-AFP migrated only as 4S after the KCl was removed, that it was not incorporated into an 8S complex, and that it was complexed by anti-AFP serum. The NGS control had not been incubated with 5 nM *E prior to desalting by gel filtration. After gamma spectrometry the TE buffer (O——O) SDG fractions (+*E) and the NGS (□——□) fractions (−*E) were counted in a liquid scintillation spectrometer. The "^{3}H" profiles and the profile of the calculated difference between the two (▲——▲) are shown in (C). The NGS profile contained only [^{125}I]-AFP and thus represents spill-over of [^{125}I] in the ^{3}H channel. The difference between the TE buffer and NGS profiles is taken to represent the *E profile of ^{3}H in samples containing both ^{3}H and [^{125}I]. By subtraction, the 4S peak in the TE buffer profile is due not to bound *E but to [^{125}I]-AFP. The removal of KCl is affirmed in (C) by the presence of *E in the 8S and heavier aggregates rather than the 4S region.

[^{125}I]-AFP (2.2 ng/ml) was incubated with 5 nM *E. The samples (0.27 ml, 1 U) were incubated with 0.03 ml of either TE buffer, normal goat serum (NGS, 1:100 final dilution) or anti-AFP (1:100 final dilution) at 0–4°C for 3 h. The [^{125}I]-AFP was observed to migrate as 4S in SDG containing 0.4 M KCl in the TE buffer and NGS controls but was complexed and precipitated only by anti-AFP. KCl was removed from duplicate samples (0.54 ml, 2 U) by gel filtration, the 4 peak fractions (void volume) eluates pooled, and aliquots (0.27 ml, 0.3 U) incubated with either TE buffer, NGS or anti-AFP, and analyzed by SDG. In the desalted cytosol containing TE buffer or NGS, [^{125}I]-AFP migrated as 4S; in the preparation containing anti-AFP it sedimented more rapidly and was pelleted at the bottom of the gradient. [^{125}I]-AFP was not incorporated into the 8S complex when the KCl was removed. The desalted NGS control which contained [^{125}I]-AFP but no *E, and the desalted TE buffer control which contained both [^{125}I]-AFP and *E were counted for ^{3}H in a liquid scintillation counter after being counted for ^{125}I in a gamma counter. The "^{3}H" radioactivity measured in the samples with no *E thus represented spillover of ^{125}I and was subtracted from samples containing both ^{125}I and *E. The difference between the two profiles represented the *E in the samples containing both isotopes. In desalted cytosol in which [^{125}I]-AFP migrated only as 4S, *E was bound in the 8S or heavier region, but not in the 4S.

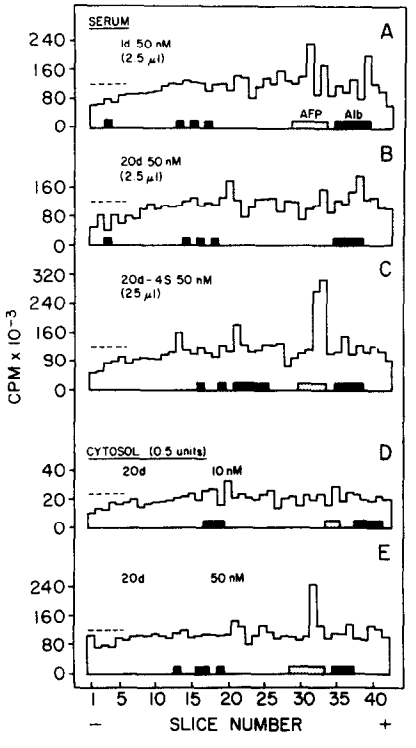

Fig. 4. Electrophoretic identification of AFP as the secondary estrogen-binding component in immature rat uterine cytosol. Samples were incubated with 10 or 50 nM *E (0–4°C × 2 h), aliquots containing 5% sucrose (w/v) layered on top of vertical 6.5% polyacrylamide disc gels (5 × 70 mm), pH 8.9 containing equimolar *E, and steady-state electrophoresis performed at 4°C (70 min) with constant current (4 mA/gel). Protein was stained with 1% amido black, or gels were sliced (1.5 mm) and radioactivity measured. At 10 nM *E, control gel slices yielded 25 ± 3.8 × 10^3 c.p.m.; at 50 nM 120 ± 13.1 × 10^3 c.p.m. The mean background c.p.m. from control gels are indicated by dotted lines. The horizontal bars indicate the location of stained proteins. (A) SPAGE of 0.05 ml of serum (1 day) diluted (1:20) with TE buffer (equivalent to 2.5 µl of undiluted serum) and incubated with 50 nM *E. (B) SPAGE of 0.05 ml of serum (20 day) diluted (1:20) with TE buffer (equivalent to 2.5 µl of undiluted serum) and incubated with 50 nM *E. (C) SPAGE of serum (20 day) 4S. Serum (0.3 ml) was incubated with 50 nM *E, subjected to SDG, and the radioactivity in 0.05 ml of each 0.2 ml fraction measured; the remainder of the 3 fractions comprising the 4S region were combined and an aliquot (0.11 ml) equivalent to 25 µl of undiluted serum 4S examined by SPAGE. (D) SPAGE of TE buffered cytosol (20 day, 0.5 U/gel) incubated with 10 nM *E. (E) SPAGE of TE buffered cytosol (20 day, 0.5 U/gel) incubated with 50 nM *E.

Electrophoretic identification of AFP as the secondary estrogen-binding component in immature rat uterine cytosol

SPAGE was utilized to characterize the 4S estrogen-binding region in uterine cytosol of the 20 day old rat (Fig. 4). At 50 nM *E 1.5 mm gel slices yielded 120 ± 13.1 ($\bar{X}$ ± S.E.M.) × 10^3 c.p.m.; at 10 nM, 25 ± 3.8 × 10^3 c.p.m. Stainable protein bands corresponding to albumin (Alb) were visible in gels after SPAGE of sera (1:20 dilution, 0.05 ml) from both 1 and 20 day old rats, whereas an AFP band was visible only at 1 day. Since SPAGE of concentrated 20 day serum (undiluted, 0.025 ml) resulted in protein overload of the gels (smearing), undiluted 20 day serum was centrifuged in SDG and an aliquot of the pooled 4S fractions (equivalent to 0.025 ml of serum) was electrophoresed ("20 day-4S"). An AFP band was then visible after staining. In the 1 and 20 day gel preparations (50 nM *E) small but significant peaks of *E corresponded to AFP. Cytosol (0.5 U/gel, 20 day) incubated at 10 or 50 nM *E and electrophoresed at equimolar *E concentrations contained both AFP and Alb. At 10 nM *E, at which 8S is observed in SDG, no significant *E peak was observed in the gel after SPAGE. At 50 nM *E, at which the same 8S and a large 4S are observed in SDG, a large *E peak associated with AFP in the gel was apparent after SPAGE.

DISCUSSION

A new model for the mechanism of action of estrogens has been proposed in which the cytoplasmic "receptor" of the immature rat uterus is equivalent to alpha-fetoprotein [5]. This model, however, does not account for the presence of a cytoplasmic estrogen receptor in the adult rat uterus in which AFP is undetectable (Linkie and LaBarbera, unpublished observations). AFP is a serum estrogen-binding protein which migrates in the 4S region in sucrose density gradients [3, 4] and declines from levels

greater than 700,000 ng/ml in the serum of 21 day old rats to 20–40 ng/ml in adulthood [14]. Therefore, serum AFP represents a possible source of contamination in the soluble supernatant of a uterine homogenate. This is especially significant in the immature animal in view of the potential estrogen binding capacity of AFP.

It was suggested that the AFP in cytosol functions as a component of an 8S macromolecular complex which has an affinity for estradiol 20 times greater than the AFP of serum origin [5]. Thus, *E binding in the 8S region should increase as the *E concentration is increased. However, evidence presented here shows that the 4S component and not the 8S form binds more *E as the *E concentration is increased, so that at 1000 nM the 4S *E complexes approximate the AFP binding capacity as estimated by radioimmunoassay (Fig. 1). Indeed comparison of the distribution of *E binding between 8S and 4S at 5 nM and 500 nM *E reveals that the two components have markedly different affinities and capacities for *E.

The 8S estrogen receptor is themolabile [1] whereas AFP is not [13]. In these experiments 8S binding predictably was abolished by temperature elevation and there was no concomitant increase in 4S binding (Fig. 2). If AFP was a binding component of the 8S complex, an increase in the 4S region after heating would be anticipated. In the same experiment, exogenous AFP bound the same amount of estradiol before and after heating, migrated only as 4S, and did not compete with the 8S component for estradiol, contrary to the report of Uriel *et al.* utilizing much less AFP and an equivalent *E concentration [5] as reported in these studies.

The possibility that AFP complexed with an intracellular component (receptor?) to sediment as 8S rather than 4S in hypotonic cytosol was tested by attempted incorporation of [^{125}I]-AFP into the 8S form (Fig. 3). [^{125}I]-AFP was added to high salt (0.4 M KCl) *E-labeled cytosol in which all estrogen-binding components exist in the 4S form. After gel filtration to remove the KCl, SDG indicated that the [^{125}I]-AFP remained in the 4S region of the gradient whereas *E migrated solely as 8S. If the 5 nM-*E-labeled 8S had consisted of AFP there would have been equilibration between unlabeled and labeled AFP after disaggregation of the 8S by the addition of KCl. Upon removal of KCl and reassociation of the binding components, the labeled AFP would be incorporated into the 8S complex. The failure of the [^{125}I]-AFP to be incorporated into 8S is taken to indicate that AFP was neither a native nor an artifactual component of the cytoplasmic 8S complex.

Other investigators have used immunoadsorbents to show that the cytosol 4S estrogen-binding component is AFP [3], an observation confirmed by use of SPAGE in which sample and gel contain equimolar concentrations of *E (Fig. 4). Conventional SPAGE proved unsuccessful as the *E either dissociated from the binding protein or the latter was labile for the conditions used for electrophoresis. Hypotonic cytosol 8S complexes did not enter gels in contrast to 4S components. At 10 nM, 8S rather than 4S was labeled in contrast to the labeling in both regions observed at 50 nM. Therefore, it was concluded that the peak of radioactivity in the 50 nM *E SPAGE gel represented the 4S estrogen-binding protein, which coincided with the stainable AFP band. Thus, AFP represents the 4S estrogen-binding component.

In summary, the results presented here do not support the assertion that AFP is the major high affinity estrogen binder in immature rat uterine cytosol. It is concluded instead that AFP is the secondary estrogen binder which comigrates with albumin in SDG as a 4S form.

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