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Biochemistry

Alpha-fetoprotein: The major high-affinity estrogen binder in rat uterine cytosols

(rat alpha-fetoprotein/estrogen receptors)

JOSÉ URIEL, DANIELLE BOUILLON, CLAUDE AUSSEL, AND MICHELLE DUPIERS

Institut de Recherches Scientifiques sur le Cancer, Boîte Postale No. 8, 94800 Villejuif, France

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ABSTRACT **Evidence is presented that alpha-fetoprotein (AFP), a serum globulin, accounts mainly, if not entirely, for the high estrogen-binding properties of uterine cytosols from immature rats. By the use of specific immunoadsorbents to AFP and by competitive assays with unlabeled steroids and pure AFP, it has been demonstrated that in hypotonic cytosols AFP is present partly as free protein with a sedimentation coefficient of about 4–5 S and partly in association with some intracellular constituent(s) to form an 8S estrogen-binding entity. The AFP → 8S transformation results in a loss of antigenic reactivity to antibodies against AFP and a significant change in binding specificity. This change in binding specificity is manifested by an increase in binding affinity for estradiol, estriol, diethylstilbestrol, and nafoxidine (a nonsteroidal anti-estrogen), and by a concomitant decrease in estrone binding. Both the antigenic and binding properties of native AFP are recovered after dissociation of the 8S complex in 0.4 M KCl. An AFP-mediated mechanism of early intracellular events associated with estrogen entry in target cells is suggested and discussed with regard to current views on steroid action.**

Alpha-fetoprotein (AFP) is the first α-globulin to appear in mammalian sera during embryonic development and the dominant serum protein in early embryonic life. Rat and mouse, as well as human AFP, possess high estrogen-binding affinity as demonstrated by immunological (1) and biochemical (2–4) methods. The total binding capacity of AFP in serum and other biological fluids differs greatly from one species to another, however, because of the coexistence in variable proportions of two molecular species of AFP, only one of which possesses high estrophylic properties (4). Rat AFP is also designated by some authors as the estrogen-binding plasma protein or EBP (5).

Kinetic data concerning the influence of aging on the serum concentration of rat AFP have shown (6, 7) that concentrations as high as several milligrams per milliliter at birth fall to less than 50 ng/ml from the 6th to 10th week of age and remain at this level in normal male and female adult animals. Increased values of serum AFP reappear in pregnant rats (7, 8) and in adult animals bearing primary liver cancer (9). In immature rats, serum AFP ranges from about 1 mg/ml (21-day-old animals) to several hundred μg/ml (25-day-old animals).

Numerous studies, reviewed recently (10, 11), have demonstrated the presence of high-affinity hormone binders, called "receptor" proteins, in soluble homogenates of estrogen-responsive tissues. Rat-uterine cytoplasmic extracts (cytosols) incubated with tritiated estrogens and subjected to density gradient centrifugation have revealed two major macromolecular complexes with sedimentation coefficients close to 4–5 S and 8–9 S; these are currently referred to as the 4–5S and the 8S "receptors." The 8S complex predominates in hypotonic solutions, whereas in salt concentrations above 0.2 M the 4S complex is by far the major binding entity.

The relatively high levels of serum AFP in immature rats prompted us to explore the contribution of AFP to the estrogen-binding capacity of uterine homogenates. The results obtained with specific anti-AFP immunoadsorbents (12, 13) provided evidence that at low salt concentrations, AFP accounts for most of the estrogen-binding capacity associated with the 4–5S macromolecular complex. It was concluded that the 4–5S entity and AFP were identical and that the presence of AFP in uterine cytosols was probably due to serum contamination.

We report here further observations which strongly suggest that the other macromolecular complex, the 8S entity, is also made up of AFP in reversible association with some intracellular constituent(s). Immunologic data and competitive assays with unlabeled estrogens and anti-estrogens have also enabled us to demonstrate significant changes in antigenicity and binding specificity of native serum AFP upon its transition to the 8S form and the reversion to the original properties of native AFP after the 8S → 4–5S transformation in hypertonic solutions.

MATERIAL AND METHODS

Animals. Female Wistar rats were used in all experiments. Amniotic fluids were obtained by puncturing the amniotic sacs of animals on the 17th to 19th day of pregnancy. The fluids were pooled, treated with charcoal (2 mg/ml) for 20 min at 37° with mild agitation, centrifuged at 5000 rpm (4000 × *g*), and stored at −80°. Uteri from immature (21- to 25-day-old) animals were removed immediately after sacrifice, cleaned of fat and connective tissue, washed for 15 min in neutral buffered saline at 0–2°, and used at once for tissue fractionation.

Tissue Fractionation. All operations were performed at ~0–2°. Five uteri were homogenized per ml of 10 mM Tris–2 mM Na_2EDTA–2 mM mercaptoethanol, pH 7.4 (TE buffer), in a polytron PT.10 Brinkmann instrument (three 5 sec bursts at a power setting of 4 and at 20 sec intervals). The homogenate was centrifuged at 180,000 × *g* for 30 min and the supernatant obtained is referred to as TE-buffered cytosol. The latter, made 0.4 M in KCl by the addition of solid KCl, is designated TEK-buffered cytosol. Immunological quantitation (see below) of AFP in the cytosols studied gave values between 2 and 10 μg/ml. The total protein content was about 2 mg/ml.

Biochemicals. Rat AFP was isolated from amniotic fluid by affinity chromatography with specific immunoadsorbents as described previously (3). Standard solutions of AFP (1 mg/ml in 0.15 M NaCl) were stored at −30°. Quantification of rat AFP was carried out by electroimmunodiffusion

Abbreviation: AFP, alpha-fetoprotein.

in agarose gels (14) with specific rabbit antisera and the double antibody technique of Guesdon and Avrameas (15). The smallest concentration of protein measurable with this technique was 50 ng/ml. Other protein determinations were done with a biuret technique in which bovine-serum albumin was used as a standard.

[2,4,6,7-^{3}H]Estradiol-17β (100 Ci/mol) was purchased from the Radiochemical Center, Amersham, U.K.; diethylstilbestrol and the unlabeled steroids, estradiol-17β, estrone, and estriol, from Roussel-UCLAF (France). The nonsteroidal anti-estrogen nafoxidine (Upjohn Co., U.S.A.) was a gift of Dr. Milgrom (Lab. Hormones, Bicêtre, France). Biogel P 300 beads were from Bio-Rad (U.S.A.) and agarose powder was from Industrie Biologique Française (Genevilliers, France). All other chemicals were of analytic grade.

Immunochemicals. Rabbit and sheep specific antisera to rat AFP and rabbit antiserum to adult rat serum proteins were prepared according to immunization procedures previously described (8). Sheep antibodies to rat AFP were isolated by adsorption of the whole antiserum on an unsolubilized polymer of rat amniotic fluid and subsequent elution of the specific antibodies at acid pH was according to the procedure of Avrameas and Ternynck (16).

A specific immunoadsorbent to rat AFP (referred to in the *text* as "Bio-gel-anti-AFP") was prepared by coupling the isolated sheep anti-AFP antibodies to glutaraldehyde-activated Bio-gel P 300 beads according to the method described by Ternynck and Avrameas (17). Pure sheep gamma globulins were coupled by the same method to Bio-gel P 300 beads (designated in the *text* as "Bio-gel-γG") and used as a negative control. The quantity of gamma globulins or antibodies coupled ranged between 1 and 1.5 mg/ml of packed beads. Beads were stored at 4° in a 10-fold volume of 0.15 M NaCl containing 0.02% sodium azide.

Immunoadsorption assays with Bio-gel-anti-AFP or control treatments with Bio-gel-γG were carried out at 2–5° in the following manner. One milliliter of the bead suspension (equivalent to 0.1 ml of packed beads) was placed in a 5-ml plastic assay tube and centrifuged at 800 × *g* for 10 min, and the supernatant was decanted and replaced by 1 ml of the desired buffer. The beads were suspended and gently stirred in the buffer solution for 30 min, centrifuged again at 800 × *g*, and the supernatant was discarded. One aliquot (0.5–1 ml) of a cytosol preparation was added to the pelleted beads and left for 2–4 hr with frequent stirring. After a final centrifugation at 800 × *g* for 10 min, the supernatant was recovered and used for subsequent treatments (see below).

Estrogen Binding. Samples of either TE- or TEK-buffered cytosols were added to glass tubes containing the appropriate quantity of [^{3}H]estradiol to make a final 5–7 nM solution and were incubated at 0–2° for 60–90 min. In competitive assays with unlabeled estrogens and anti-estrogens, the compounds were placed in the glass tubes at the same time as the labeled estradiol. A sample of rat AFP (10 μg/ml) in TEK buffer was simultaneously incubated in 5 nM [^{3}H]estradiol and served as a reference standard in density gradient centrifugations.

Density Gradient Centrifugation. Aliquots (0.2–0.3 ml) of the samples were layered on top of either linear 5–35% glycerol or 5–20% sucrose gradients (3.8 ml) in TE or TEK buffers and centrifuged at 50,000 rpm for 15–16 hr at 2° in a L3-50 Spinco ultracentrifuge in a SW-50.1 rotor. Estradiol-labeled rat AFP was run in a separate gradient. Two-drop fractions were collected after centrifugation. After the addition of 5 ml of a dioxane-based scintillation fluid to each fraction, the bound radioactivity was counted in an "Intertechnique" S2-40 liquid scintillation spectrometer with an efficiency of 35% for tritium.

The apparent sedimentation coefficients of the radioactive peaks were calculated according to Martin and Ames (18) with use of a preparation of rat AFP with a sedimentation coefficient of 4.5 S as a marker (3).

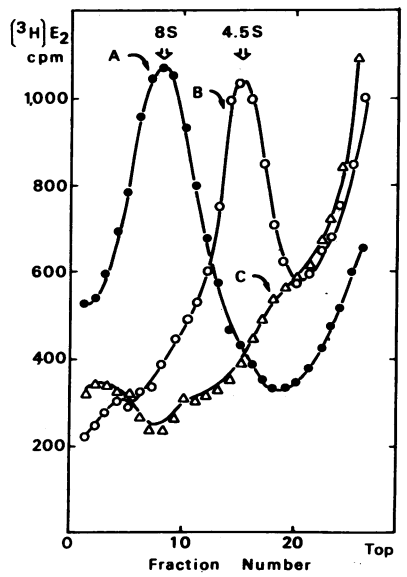

FIG. 1. Density gradient patterns of uterine cytosol from 21-day-old rats. Effect of anti-AFP antibodies on the estradiol (E_2)-binding capacity after 8S → 4–5S transformation in 0.4 M KCl. The cytosol (10 uteri per 2 ml) was prepared in TE buffer and immunoadsorbed with Bio-gel anti-AFP antibodies as described under *Material and Methods*. One portion (0.2 ml) was incubated for 60 min with 5 nM [^{3}H]estradiol and centrifuged for 16 hr through a 5–35% glycerol gradient prepared in TE buffer (pattern A). The rest of the cytosol was made 0.4 M in KCl by the addition of the appropriate quantity of solid salt. One half of the solution was treated with Bio-gel γG beads and served as a control, whereas the other half was immunoadsorbed again with Bio-gel anti-AFP beads. Aliquots (0.2 ml) of the control and immunoadsorbed preparations were incubated for 60 min in 5 nM [^{3}H]estradiol and centrifuged for 16 hr in 5–20% sucrose gradients containing TEK buffer (patterns B and C, respectively).

RESULTS

Identification of AFP in the 8S estrogen-binding constituent of uterine cytosol

The identification of AFP in the 4–5S estrogen-binding complex of TE-buffered uterine cytosols and the ineffectiveness of anti-AFP antibodies to reduce estradiol binding to the 8S macromolecular entity were reported previously (12, 13). However, the experiment illustrated in Fig. 1 enabled us to demonstrate the presence of AFP associated with the 8S macromolecular entity. Two milliliters of a preparation of TE-buffered cytosol (AFP content 8.2 μg/ml) from 21-day-old rats was first treated with Bio-gel-anti-AFP beads (see *Material and Methods*) to remove free AFP. An aliquot (1.6 ml) of the immunoadsorbed cytosol was made 0.4 M in KCl by the addition of the appropriate quantity of solid KCl (TEK-buffered cytosol). This operation is known to dissociate the 8S macromolecular complex into a smaller (4–5S) estrogen-binding constituent. The TEK-buffered cytosol was then divided in two equal portions; one was immunoadsorbed again with Bio-gel-anti-AFP, whereas the other was

Table 1. Physicochemical data and competition of nonradioactive compounds with [^{3}H]estradiol

Entity	Molecular weight	K_a (E_2)* (10^8 M^{-1})	Anti-AFP†	% Reduced [^{3}H]estradiol binding‡				
				E_2	E_1	E_3	DES	UA
4-5S	76,200 (23)	~0.4 (13)	+	100	120	40	41	25
AFP	72,000 (3)	0.6–1.4 (3,24)	+	100	118	42	40	35
8S	~240,000 (11)	14 (25)	—	100	82	80	98	70

Numbers in parentheses are reference numbers.
* Association constant for estradiol-17β.
† Reactivity toward anti-AFP antibodies.
‡ Estimates of competitive effect were taken as the differences in the areas under the peaks (Fig. 3) obtained after labeling with 7 nM [^{3}H]estradiol alone and in the presence of a 12-fold excess of unlabeled compounds. Competition is expressed as the percentage of reduced [^{3}H]estradiol binding, the inhibition effect of nonradioactive estradiol-17β taken as 100. Values are the average of three experiments with uterine cytosols from 21- to 25-day-old rats. E_2, estradiol-17β; E_1, estrone; E_3, estriol; DES, diethylstilbestrol; UA, nafoxidine.

treated in a similar manner with Bio-gel-γG beads and served as a control. Aliquots (0.2 ml) of the immunoadsorbed TE- and TEK-buffered cytosols and of the control sample were incubated with 5 nM [^{3}H]estradiol and centrifuged in a sucrose gradient and the radioactivity counted. The AFP content of these preparations, measured as described in *Material and Methods*, was 8.20, 0.14, and 1.84 μg/ml, respectively.

The results illustrated in Fig. 1 show that when the 8S macromolecular complex (pattern A) was dissociated at a high salt concentration (0.4 M KCl), most of the radioactivity bound to the newly formed 4–5S complex (pattern B) disappeared when immunoadsorbed with anti-AFP antibodies (pattern C). This suggests that at low salt concentration, part of the AFP in uterine cytosols is present in a free form that has a sedimentation coefficient of about 4–5 S and part is associated reversibly with some intracellular constituent(s) to form the 8S estrogen-binding entity. It is interesting to note that in such a complexed form the AFP retains its estradiol-binding affinity, whereas its antigenic properties appear severely modified as evidenced by its inability to react with specific antibodies. Inability to react with specific antibodies was confirmed by an assay of AFP in the fractions collected after density gradient centrifugation in TE buffer of a uterine cytosol from 22-day-old rats. The results, in agreement with previous data obtained with uterine cytosol from 10-day-old animals (12), confirm the presence of free AFP as a single peak restricted to the 4–5S zone of bound radioactivity. No AFP could be immunologically detected in any other fraction of the gradient, including the 8S zone.

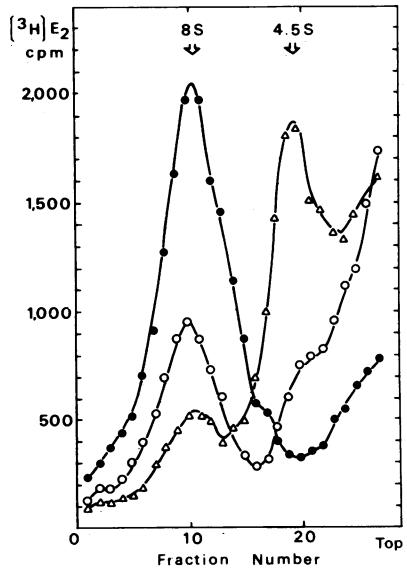

FIG. 2. Density gradient patterns of uterine cytosol (22-day-old rats) in TE buffer. Effect of exogenous AFP on the radioactivity bound to the 8S macromolecular complex. To aliquots (0.2 ml) of a uterine cytosol (5 equivalent uteri per ml) were added 0.05 ml of TE buffer containing 0 (●), 6.5 (○), and 19.5 pmol (△) of AFP. The samples were incubated for 60 min in 5 nM [^{3}H]estradiol (E_2), layered on top of linear 5–35% glycerol gradient in TE buffer, and centrifuged at 50,000 rpm for 16 hr at 2° in a Spinco L3-50 ultracentrifuge with a SW-50.1 rotor. Immunologic quantitation of endogenous free AFP in the cytosol preparation revealed a concentration of 110 pmol/ml (22.1 pmol/0.2 ml aliquot).

Effect of exogenous AFP

We investigated the effect of exogenous AFP on the sedimentation profiles obtained after incubation with radiolabeled estradiol by adding pure AFP from rat amniotic fluid at various concentrations to aliquots (0.2 ml) of a uterine cytosol in TE buffer (endogenous free AFP content 22.1 pmol per aliquot). Fig. 2 illustrates the competitive effect of exogenous AFP on the radioactivity bound to the 8S macromolecular complex and the concomitant appearance in the 4–5S zone of a peak of radioactivity that increases at the same time with the amount of added AFP.

Inhibition of about 80% of the radioactivity bound to the 8S complex was observed in the presence of approximately 41 pmol of AFP per uterine equivalent (22.1 pmol of endogenous free AFP plus 19 pmol of added AFP). This represents a good correlation between free AFP content and the extent of competitive binding, considering that the concentration of estradiol-binding sites in the 8S complex is generally estimated at about 1.4 pmol per uterus (19), and that the binding affinity for estradiol of the 8S complex is 10- to 20-fold higher than that for AFP (see Table 1).

Binding specificity: competitive studies

It has been previously reported that the affinity of the 8S macromolecular complex in uterine cytosol from 10-day-old rats is higher for estradiol than for estrone, whereas the reverse occurs with the 4–5S complex (13). We have explored further the binding specificity of these two macromolecular complexes in uterine cytosols from older rats and have compared it to that of AFP. Samples of the same cytosol in hypotonic (TE-buffered) and hypertonic (TEK-buffered) media and samples of rat AFP (10 μg/ml) were incubated in 7 nM [^{3}H]estradiol alone or in combination with a 12-fold excess of nonradioactive estradiol-17β, estrone, estriol, or diethyl-

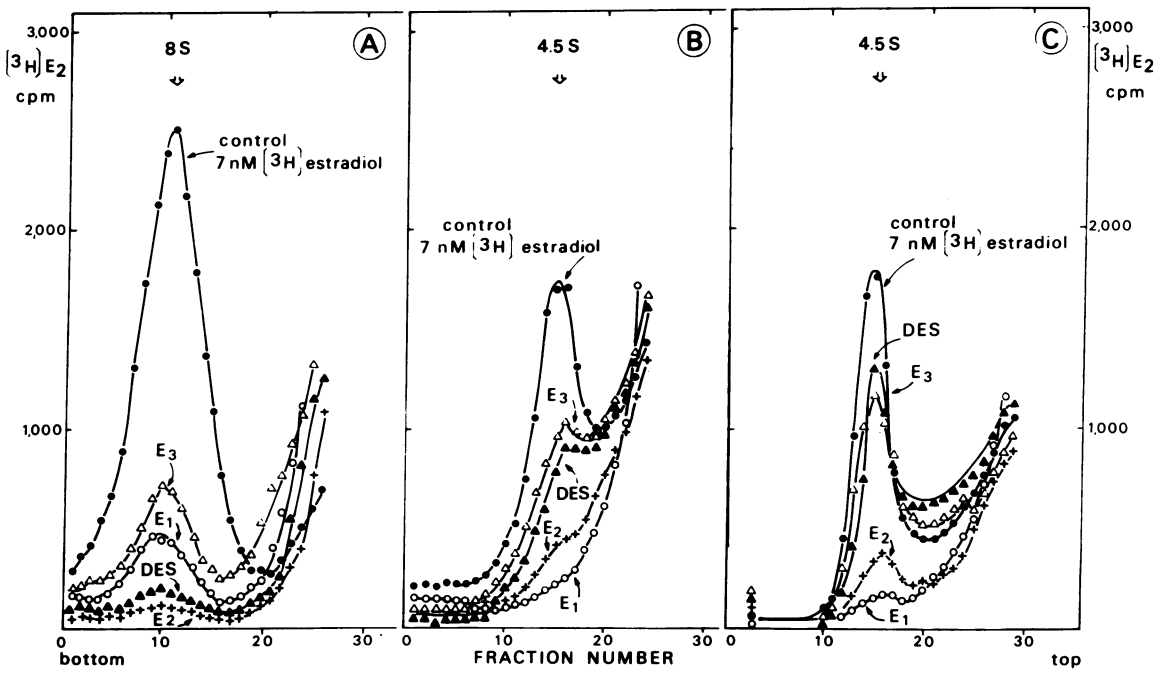

FIG. 3. Density gradient patterns of uterine cytosol and rat AFP. Competitive effect of unlabeled compounds on [^{3}H]estradiol binding capacity. Samples of the same uterine cytosol from 25-day-old rats in TE buffer (pattern A) and TEK buffer (pattern B) and a sample of rat AFP (10 μg/ml in TEK buffer) were incubated for 90 min in 7 nM [^{3}H]estradiol alone (controls) or in combination with a 12-fold excess of estradiol-17β (E_2), estrone (E_1), estriol (E_3), or diethylstilbestrol (DES). Aliquots (0.2 ml) were layered on top of 5–35% glycerol gradients (TE-buffered cytosol) or 5–20% sucrose gradients (TEK-buffered cytosol and AFP) and centrifuged for 15 hr as indicated in *Material and Methods*.

stilbestrol. After density gradient centrifugation of 0.2 ml aliquots, the radioactivity bound to the 8S, 4–5S, and AFP peaks was measured. The degree of competitive inhibition by nonradioactive compounds was expressed as the percentage of reduced [^{3}H]estradiol binding. From an analysis of the radioactivity patterns represented in Fig. 3 and the data plotted in Table 1, it clearly appears that the following sequence, estradiol-17β > diethylstilbestrol > estrone > estriol, characterizes the binding affinity of the 8S entity. After the 8S → 4–5S transformation in 0.4 M KCl, the affinity order changes to estrone > estradiol > diethylstilbestrol ≃ estriol, a sequence similar to that found for rat AFP.

Competitive assays of the type described above were also carried out with the nonsteroidal anti-estrogen nafoxidine, which is known to compete with estradiol for binding sites in uterine cytosols, both *in vivo* (20) and *in vitro* (21, 22). The results obtained (Table 1) confirm the great effectiveness of nafoxidine in reducing estradiol uptake by the 8S complex. However, rat AFP or the 4-5S complex was less effective in inhibiting the rate of estradiol binding in the presence of nafoxidine. In contrast, Rochefort *et al.* (21), using a different technique than ours, have reported identical effects of nafoxidine in competitive assays with estradiol on both the 8S and 4–5S entities.

Table 1 summarizes the results presented here and incorporates some physiochemical data taken from the literature. The conclusion can be drawn that after the 8S → 4–5S transformation that occurs upon addition of KCl to 0.4 M, the physicochemical, antigenic, and estrogen-binding properties of the 8S complex change in such a way that the properties of the newly formed 4–5S complex strongly resemble those of AFP. This serum protein appears then as the major if not the unique high-affinity estrogen binder in uterine cytosol from immature rats.

DISCUSSION

During the past 10 years, research on some aspects of the mechanism of action of steroid hormones has led to the notion of a "two-step interaction mechanism" (26, 27). Briefly, upon entering the cell the hormone associates first with a cytoplasmic receptor protein(s), the 8S entity; this is followed by a temperature-dependent transformation of the initial complex and its translocation to the nucleus, where it can be then identified as a 5S binding entity. Whether this 5S nuclear receptor represents the last steroid–protein complex before initiation of hormonal action in the nucleus or whether it is only another intermediate binder that finally delivers the hormone to specific nuclear sites remains to be elucidated.

The evidence presented here, that AFP, a plasma α-globulin, accounts mainly if not entirely for the high affinity estrogen-binding properties of rat uterine cytosols, has obvious implications on current views of the mechanism of early action of estrogenic hormones with target cells. Although the existence of cytoplasmic estrogen "receptors" becomes questionable, the differences in antigenicity, sedimentation coefficient, and binding specificity observed between native AFP and the 8S cytoplasmic entity strongly suggest that the estrogen–AFP complex associates with some cytoplasmic acceptor(s) to form the 8S entity. In addition to its reversibility, three major facts characterize this AFP → 8S transition: (*i*) hindering of the antigenic sites of AFP as evidenced by its inability to interact with specific anti-AFP antibodies; (*ii*) a considerable increase in the sedimentation coefficient; and (*iii*) some conformational change that results in a higher binding affinity for estradiol, diethylstilbestrol, and estriol and a concomitant lowering of affinity for estrone. The transition is reversible at high salt concentrations where the initial properties of native AFP seem to be recovered. Although

the ionic strength of the different intracellular microcompartments is unknown, it is likely that under more physiological conditions AFP may form a spectrum of macromolecular complexes with modulated estrogen-binding properties.

As far as the relationship of AFP to the 5S nuclear entity, (the so-called "nuclear estrogen receptor") is concerned, preliminary assays have shown no detectable AFP in nuclear myofibrilar homogenates from rat uteri. Whether this result reflects the true absence of AFP in the nuclear compartment (the limit of sensitivity of the method used is about 40 ng/ml) or rather the inability of the 5S complex, like the 8S cytoplasmic entity, to react with specific antibodies necessitates further investigation. However, it is generally accepted that the 5S nuclear entity derives from the 4 to 5S cytoplasmic entity upon temperature-dependent transformation in the presence of estradiol (27). On this basis one might propose that the 5S nuclear entity may be an association of AFP with some cytoplasmic or nuclear constituent that confers to the transformed molecule a new conformational state and different sedimentation properties.

Another relevant question brought up by the present work concerns the manner in which estrogenic hormones enter target cells. It has generally been assumed that the entry proceeds by nonspecific free diffusion. The proposed AFP-estrogen carrying process represents a different alternative. It can be related to observations reported recently by Milgrom and his coworkers (28), who have indicated the possibility that estrogen entry into uterine cells might be a protein-mediated mechanism acting as an intermediate step between the uptake of estrogens from the plasma and their delivery to the conventional "cytoplasmic receptor." In support of this conclusion, these authors noticed that [^{3}H]estradiol entry was greatly inhibited by unlabeled estradiol but much less inhibited by diethylstilbestrol in spite of the fact that the 8S entity shows similar binding affinity for these estrogens. They also noted saturability of estradiol entry with an apparent affinity corresponding to a K_d about 5 nM, whereas the K_d of the 8S entity was about 0.5 nM. It is clear that these data are among the differences observed in the present work between AFP and the 8S entity and can be explained in terms of the AFP $\rightarrow$ 8S transition outlined above: AFP possesses lower affinity for diethylstilbestrol than for estradiol and binds the latter with an affinity constant 10- to 20-fold below that of the 8S entity.

In addition, the AFP-mediated mechanism of estrogen activity implies some regulatory step at the intravascular compartment because the AFP–estrogen complex is a macromolecular entity. This focuses attention on earlier work (29) concerning events, other than protein binding, elicited by estrogen action in the uterus, e.g., acute hyperemia, edema, histamine release, increased cell permeability. This battery of very early estrogen-induced responses may be a pivotal step in intimate relation with AFP-estrogen entry into the cell and its subsequent 8S transformation.

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