Estrogen Binding Proteins of Calf Uterus. Inhibition of Aggregation and Dissociation of Receptor by Chemical Perturbation with NaSCN†

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ABSTRACT: Sodium thiocyanate up to 0.5 M is compatible with a stable estradiol-*t*-receptor complex during sucrose gradient centrifugation; however, the maximum permissible concentration is 0.1 M during Sephadex G-100 and G-200 chromatography. When NaSCN 0.1 M is added to low-salt cytosol (~7 mg of protein/ml): (1) age-dependent aggregation of receptor is inhibited; (2) peaks of estrogen-binding activity in sucrose gradients and on Sephadex chromatography are sharp; (3) instead of the usual larger molecular states ("8S") found in low salt, most of estrogen receptor is under the following form: 4.1S; Stokes radius, 36 Å; mol wt 61 000; f/f_0, 1.25; homogeneous at electrofocusing, with isoelectric point at 6.0. When cytosol containing NaSCN 0.1 M is diluted down to 2–3 mg of protein/ml or, only for sucrose gradients, NaSCN concentration is increased to 0.4–0.5 M, the 61 000 dalton species decreases, being substituted, without loss of bound estradiol-*t*, by the following estrogen-binding entity: 2.8S; Stokes radius, 28 Å; mol wt 32 000; f/f_0, 1.44. In the presence of NaSCN, KCl up to 0.4 M does not affect in a significant manner the molecular properties of the above forms. When NaSCN is dialyzed out, most receptor reverts to a 8–9S state. When cytosol is preincubated with Ca^{2+} (4 mM) and KCl (0.4 M) before addition of NaSCN, the above picture is modified only in the following aspects: (1) Sephadex chromatography peaks are broader and slightly but reproducibly shifted toward higher elution volumes; (2) the electrofocusing pattern consists of a two-peak heterogeneous band shifted toward higher pH (isoelectric points, 6.4 and 6.6); (3) upon dialysis of NaSCN there is little or no reversion to faster sedimenting states. These modifications appear to depend on limited proteolytic attack of receptor by Ca^{2+}-activated receptor transforming factor (RTF), not on binding of Ca^{2+} to receptor. Present data suggest that the 4.1S entity is a dimer resulting from side-by-side pairing of 2.8S subunits. Molecular dimension of larger receptor forms purified from cytosol are consistent with the hypothesis that under native conditions in vivo dimers are coupled end-by-end into tetrameric structures with two stronger (between subunits) and two weaker (between dimers) bonding regions, and that tetramers may further self-associate. While NaSCN reversibly releases native dimers and subunits by direct impairment of intersubunit bonds, Ca^{2+} activated RTF irreversibly and specificaly releases slightly modified, about 60 000 mol wt dimers, by preferential proteolytic attack of the weaker bonding regions and indirect destruction of involved bonds. In vivo, this effect of RTF may be instrumental in mobilization and nuclear penetration of receptor–estradiol complex. Heteroassociation of receptor with other proteins of cytosol is not excluded by the above hypothesis.

When estradiol-*t*-labeled cytosol from rat uterus homogenized with neutral buffer of low ionic strength (ionic molarity, 10^{-2} to 10^{-3}) is centrifuged, immediately after preparation, in sucrose density gradients in the same low-salt solution, the estradiol-*t*-receptor complex sediments as a discrete band at 7–9S, depending on dilution of cytosol (Toft and Gorski, 1966; Stancel et al., 1973a). Under traditionally applied conditions, sedimentation is at approximately 8S (Jensen et al., 1969), and therefore one generally refers to this state of receptor as "8S". After partial purification from calf uterus cytosol, the above receptor species sediments at 8.6S and shows a Stokes radius of 67 Å as measured by chromatography on calibrated Sephadex G-200 columns; computations based on these values give a mol wt of 238 000 and a frictional ratio of 1.65 (Puca et al., 1971).

The "8S" state of receptor is rather unstable, tending both to form larger molecular complexes with aging of cytosol, and to decompose. After storage of cytosol at 4 °C for 24 h, virtually all receptor is part of large, irreversible aggregates which sediment rapidly to the bottom of the gradient and are excluded by Sephadex G-200. Tendency toward aggregation is enhanced by acid pH, lack of reducing agents, higher protein concentration, and particular procedures like salting-out with $(NH_4)_2SO_4$ (Puca et al., 1975).

In the presence of KCl at about physiological concentrations or higher, the "8S" in crude cytosol is reversibly transformed into slower sedimenting entities: depending on dilution of cytosol and presence of reducing agents, at 0.15–0.4 M KCl receptor may sediment as discrete bands in the 5.4–4.8S or the 4.0–3.8S regions; in the presence of 4 M urea, 1 M KCl, and reducing agent, the receptor sediments at 3.6S (Stancel et al., 1973a,b). At 0.2–0.4 M KCl, the partially purified 8.6S state is reversibly transformed into an entity sedimenting at 5.3S, with a 54-Å Stokes radius as measured by exclusion chromatography; based on these parameters, a mol wt of 118 000 and a frictional ratio of 1.67 have been computed for this species (Puca et al., 1972).

The variability of sedimentation rate of receptor in cytosol is generally considered to derive mainly from changes in protein–protein interactions resulting from modifications of environmental conditions. Two basic types of interaction may be pertinent: (a) specific interaction among components of a well-defined quaternary receptor structure; (b) nonspecific aggregation with other cytosol components under artificial conditions like those introduced by homoge-

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nization. To advance in the understanding of receptor structure and function, as well as to block age-dependent aggregation of receptor in cytosol, a main obstacle in the way of purification of receptor in tangible amounts, we searched for a way to inhibit protein interactions involving receptor without inducing major conformational modifications of receptor.

Therefore, we tested chaotropic salts, i.e., salts whose anions increase the solubility of nonpolar molecules in water (Hatefi and Hanstein, 1969; Hanstein et al., 1971) and, contrary to agents like urea, guanidine hydrochloride, or detergents, produce dissociation of proteins at concentrations which do not cause major shifts in protein conformation (Sawyer and Puckridge, 1973). We have found that, when one of these salts, sodium thiocyanate, is present, not only age-dependent aggregation of receptor in cytosol is inhibited indefinitely but, also, receptor in low-salt cytosol is included in Sephadex G-200 and G-100 matrices, peaks are sharp on both Sephadex chromatography and sucrose gradients, and a previously unknown 2.8S, 32 000-dalton, estrogen-binding entity appears. Furthermore, we have investigated, in the presence of NaSCN, receptor in cytosol in which receptor transforming factor (RTF[1]) was previously activated by addition of Ca^{2+}. We present a hypothesis concerning receptor structure and receptor transformations which comprehends past and present data.

Materials and Methods

Materials. All reagents were of analytical grade. NaSCN (ACS) was purchased from C. Erba. 17β-Estradiol-*6,7-t* (40 Ci/mmol specific activity; New England Nuclear) was 97% pure at the time of the experiments. Tris was purchased from Sigma Chemical; Sephadex G-25, G-100, and G-200 from Pharmacia; DE-52 cellulose from Whatman; sucrose (ACS) from C. Erba. Reference molecules for Stokes radius (a) and sedimentation rate (s) determination were obtained: albumin (bovine plasma) (a, 35.5 Å), albumin (ex ovo) (a, 27.3 Å; s_{20}, 3.55), myoglobin (horse muscle) (a, 20.7 Å), cytochrome c (horse heart) (a, 16.4 Å), chymotrypsinogen A (cow) (a, 22.4 Å), IgG (human) (a, 53 Å), from SERVA; Blue Dextran 200 from Pharmacia; leucine-*t* (30Ci/mmol specific activity; New England Nuclear). Values of Stokes radii are from Andrews (1970). Sedimentation coefficient of ovoalbumin is from Fox and Foster (1957).

Buffers. The following buffer solutions were used: TED, pH 7.5; TEDSCN, pH 7.5 (containing 0.1 M NaSCN, except when specified otherwise). To these buffers additional salts were added when needed, as specified in text.

Sucrose gradient centrifugation (Martin and Ames, 1961) was carried out in a Beckman-Spinco L2-65 ultracentrifuge on 5–20% or 10–30% sucrose gradients, using the SW-65 Ti rotor. Preliminary tests of linearity of gradients were carried out as previously described (Puca et al., 1971). Sucrose gradients were prepared either in TED pH 7.5 buffer, or in TEDSCN pH 7.5 buffers; when specified, 0.4 M KCl was added to the above buffers.

Test samples were always in 0.1 ml of buffer solution, to which 0.1 ml of a 1% solution of albumin ex ovo (3.55S) was added as internal reference. Ovoalbumin was detected by OD at 280 nm. Separate preliminary test showed that ovoalbumin, in the amount specified above, does not interfere with sedimentation of receptor proteins. Collection of gradients was carried out as described elsewhere (Puca et al., 1971). The collected fractions were brought to 1 ml with H_2O and OD at 280 nm measured before assessing radioactivity as described later.

Gel Filtration. Standard Pharmacia K25/50, K26/100, and K50/100 columns fitted with upward flow adaptors were used. The columns were packed in one-step with the required type of Sephadex, swollen according to the instructions by Pharmacia. Column equilibration and elution were performed in a cold room at 4 °C. Total volumes of the columns were measured by Blue Dextran 2000, and the internal volumes by leucine-*t*. All other operations were carried out as described elsewhere (Puca et al., 1971).

Electrofocusing. The LKB 110-ml standard electrofocusing column equipped with double-cooling jacket was used. All operations were carried out as described elsewhere (Puca et al., 1971).

Radioactivity Assay. Toluene-phosphor solution (10 ml), containing 3.92 g % of 2,5-diphenyloxazole, 0.18 g % of *p*-bis(*o*-methylstyryl)benzene, and 330 ml of Triton X-100 in toluene, was added to counting vials containing not more than 1.0 ml of aqueous sample. Radioactivity was measured in a Mark 1 liquid scintillation spectrometer with about 35% efficiency.

Protein Assay. Protein was measured by a modification (Eggstein and Kreutz, 1955) of the method of Lowry et al., 1951. Crystalline bovine plasma albumin was used as standard. Routinely, chromatographic effluents were examined spectrophotometrically for protein, either automatically by an Uvicord II (LKB), or manually by a Zeiss PMQ 3 spectrophotometer.

Preparation of Cytosol. All operations were carried out at +4 °C, either in a cold room or in refrigerated centrifuges. Immature calf uteri weighing not more than 30 g were collected at the local slaughterhouse as soon as the animals were killed and kept in plastic bags buried in crushed ice while rushed to the laboratory. The uteri were stripped of connective tissue and either used immediately, a requirement when receptor transforming factor is needed, or stored in liquid nitrogen up to the time of use. Fresh uteri, or frozen uteri after thawing at +4 °C, were minced in a meat grinder and homogenized in 4 volumes (except when otherwise specified) of TED pH 7.5 buffer, by means of an Ultraturrax homogenizer (Janke and Kunkel, Model TP 18/2) in six runs of 15 s each, with 60-s intervals. The homogenate was centrifuged for 45 min in the Beckman-Spinco L2-65, using the rotor Ti 50 (150 000g). About 7×10^5 dpm of 17β-estradiol-*6,7-t* (specific activity 40 Ci/mmol) was added per ml of decanted supernatant, followed by incubation at 4 °C for 1 h.

Preparation of "Thiocyanate Cytosol". Routinely, cytosol just prepared using TED pH 7.5 buffer is dialysed against TEDSCN pH 7.5 buffer (which contains NaSCN (0.1 M) except when specified otherwise) for 4 h at 4 °C. This procedure has the collateral advantage of removing excess radioactive hormone. As shown later, NaSCN (0.1 M) neither interferes with binding of 17β-estradiol by receptor nor affects, for at least several weeks at 4 °C, stability of the estradiol–receptor complex once formed.

Preparation of the Ca^{2+}-RTF Receptor Form. KCl (0.4 M) and $CaCl_2$ (4 mM) were added to estradiol-*t*-labeled

[1] Abbreviations used are: RTF, receptor transforming factor; cytosol, cytoplasmic soluble fraction; TED, 10^{-2} M Tris·HCl–10^{-3} M EDTA–10^{-3} M dithiothreitol; TEDSCN, 10^{-2} M Tris·HCl–10^{-3} M EDTA–10^{-3} M dithiothreitol–10^{-1} M NaSCN; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

cytosol just prepared from fresh, nonfrozen uteri. After 1 h at +4 °C, the cytosol was dialyzed for 4 h at 4 °C against TEDSCN buffer pH 7.5.

Results

Effect of NaSCN on 17β-Estradiol-Receptor Interaction. Presently, estrogen receptor proteins can be traced only by bound radioactive hormone. Assessment of the effect of NaSCN on stability of estradiol-*t*-receptor complex is thus a prerequisite for applying this salt to the study of receptor proteins. Because other experimental conditions too are known to affect stability of the estrogen-receptor complex (Puca et al., 1971, 1972), the effect of NaSCN was tested under the actual conditions to be used for molecular and other characterizations of receptor proteins.

Estradiol-*t*-labeled, fresh uterine cytosol was prepared using TED, pH 7.5, buffer, and samples of it were dialyzed for 4 h at +4 °C against TEDSCN buffers containing different concentrations of NaSCN (0.05–0.1–0.15–0.4–0.5 M). Thereafter, the samples were either centrifuged in sucrose gradients or chromatographed on Sephadex, at concentrations of NaSCN corresponding to those of the dialyzing buffers.

Centrifugation at +4 °C in 5–20% or 10–30% sucrose gradients overnight did not produce significant dissociation of protein-bound hormone within the range of NaSCN concentrations tested. Similar results were obtained when cytosols were chromatographed at +4 °C on small Sephadex G-25 columns (i.d. 1.2 cm; total volume, 20 ml). On the contrary, when chromatography was carried out on Sephadex G-100 and G-200 columns, at +4 °C and in general under conditions to be applied later in the measurement of Stokes radii of receptor proteins (i.d. 2.6 cm; total volume, 350 ml; reverse flow; collection of about 400 ml of effluent in 15 h), only NaSCN concentrations up to 0.1 M were compatible with the stable estradiol-receptor complex (data not shown).

Also, NaSCN up to at least 0.1 M does not interfere with formation of estradiol-receptor complex and estradiol-receptor complex can be safely stored for at least 1 week in the presence of 0.1 M NaSCN (data not shown).

Characterization of Native Receptor Proteins in the Presence of NaSCN. The term "native" is used here and in previous research (Bresciani et al., 1973) to indicate receptor proteins from cytosol of immature uteri homogenized in TED buffer solutions (no Ca^{2+} added) and kept at low temperature (4 °C) at all times.

Sephadex Chromatography. When immature calf uterus is homogenized with dilute buffer solution (TED, pH 7.5) and the resulting low-salt cytosol (~7 mg of protein/ml) is labeled with estradiol-*t* and chromatographed in the same buffer on Sephadex G-200, most of the bound radioactivity is "excluded" from the gel matrix (Puca et al., 1975). If, however, NaSCN (0.1 M) is added to the above cytosol immediately after preparation and chromatography is carried out in TED buffer containing NaSCN (0.1 M), the bound radioactive hormone is eluted well after the "void" volume of the column, as shown in Figure 1. Note that in the presence of NaSCN there is virtually no excluded material, and the included peak shows a trailing shoulder. The column was calibrated, and therefore a Stokes radius of 35 Å could be computed for the included peak (apex) by comparison with K_d's of reference proteins on a Porath plot.

Having succeeded in "including" the native estradiol-receptor complex in the Sephadex G-200 matrix, we tried to

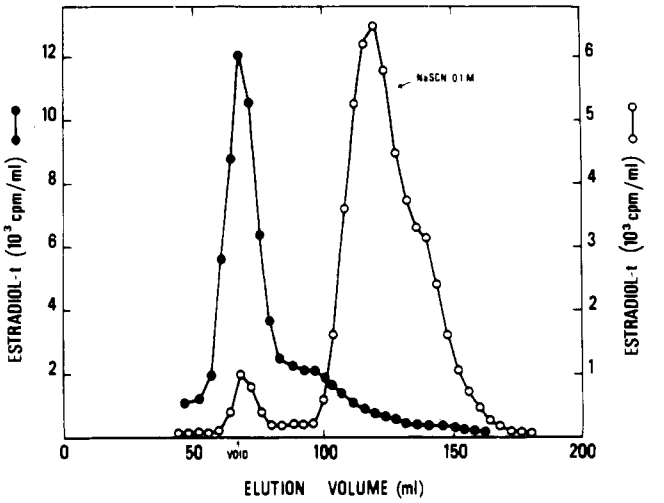

FIGURE 1: Chromatography of "low-salt" estradiol-*t*-labeled cytosol (~7 mg of protein/ml) from calf uteri homogenized with TED buffer, pH 7.5, on a calibrated column of Sephadex G-200 (●); NaSCN (0.1 M) present in cytosol and in column buffer (○). When NaSCN is present, most of protein bound estradiol-*t* is "included" in the gel matrix: K_d of peak (apex) corresponds to a molecule with a 36-Å Stokes radius (V_e = 115 ml). When NaSCN is absent, most of protein-bound hormone is "excluded" by the gel matrix: elution volume corresponds to "void" volume of the column. The column was a standard K25/50 Pharmacia. Upward flow: 14 ml/h. Fractions: 2 ml. Calibration data: V_i = 201 ml; blue dextran, V_0 = 68 ml; plasma albumin, V_e = 121 ml; chymotrypsinogen, V_e = 158 ml; cytochrome *c*, V_e = 174 ml.

improve resolution by using Sephadex G-100. As shown in Figure 2A, chromatography on Sephadex G-100 in NaSCN (0.1 M) indeed gives two included peaks, a first one with a K_d corresponding to a Stokes radius of 35 Å and a second one for which the computed Stokes radius is 28 Å. We conclude that the 1st peak corresponds to the main peak in the Sephadex G-200 chromatography, and the 2d peak to the shoulder of the main peak on G-200. Following the hypothesis that this 2d peak corresponds to a subunit originating from dissociation of the receptor form in the 1st peak, we tried to favor dissociation of this smaller entity by various means. Being barred from increasing NaSCN concentration because of dissociation of estrogen-receptor complex, we carried out chromatography at 12 °C and/or lowered the pH of the TEDSCN buffer to 6.8, without positive results. However, dilution of thiocyanate cytosol from the usual protein concentration of 6–7 mg/ml down to 2–3 mg/ml was successful; as shown in Figure 2B, the 1st peak decreased while the 2d one increased to become the major peak, without loss of total bound radioactivity. From a number of chromatographies on calibrated Sephadex G-200 and G-100 columns, the following average Stokes radii (±SE) were obtained: 35.9 (±0.7) Å for the 1st peak, and 27.8 (±0.7) Å for the 2d peak.

Sedimentation Rate. Sucrose gradient analysis was carried out in parallel to gel filtration studies. As shown in Figure 3, centrifugation of undiluted (6–7 mg of protein/ml), freshly prepared low-salt cytosol shows, as usual, a peak in the 8–9S region (Figure 3A); if, however, NaSCN 0.1 M is present, radioactivity sediments as a sharp peak at 4.1S (Figure 3B). If NaSCN concentration is increased to 0.4–0.5 M NaSCN, the 4.1S peak disappears and is quantitatively substituted by a peak at 2.8S (Figure 3C). At intermediate concentrations, both 4.1S and 2.8S peaks are found. Several separate runs gave on the average (±SE), 4.1S (±0.1) and 2.8S (±0.2) for the two peaks, using ovoal-

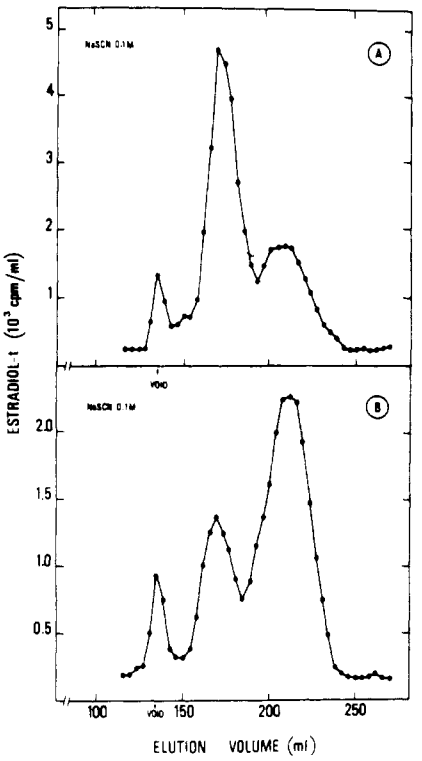

FIGURE 2: Chromatography of "low-salt" estradiol-*t*-labeled cytosol from calf uteri homogenized with TED buffer, pH 7.5, on a calibrated column of Sephadex G-100. NaSCN (0.1 M) present in both cytosol and column buffer. (A) Protein concentration of cytosol was 7 mg/ml. K_d of major peak corresponds to a molecule with a 36-Å Stokes radius (V_e = 165 ml); K_d of medium peak corresponds to a molecule with a 28-Å Stokes radius (V_e = 205 ml); minor peak is "excluded" by the gel; i.e., elution volume corresponds to "void" volume of the column. (B) Protein concentration of cytosol was 2.5 mg/ml. K_d of major peak corresponds to a molecule with a 28-Å Stokes radius (V_e = 209 ml); K_d of medium peak to a molecule with a 36-Å Stokes radius (V_e = 165 ml); minor peak is "excluded". The column was a standard K26/100 Pharmacia. Upward flow: 25 ml/h. Fractions: 4 ml. Calibration data: V_i = 431 ml; blue dextran, V_0 = 139 ml; plasma albumin, V_e = 171 ml; ovoalbumin, V_e = 201 ml; chymotrypsinogen, V_e = 258 ml; cytochrome *c*, V_e = 319 ml.

bumin (3.55S) as internal standard.

Also, sedimentation rates of the 2d peak obtained on Sephadex G-100 (see Figure 2) were checked by combining and concentrating by dialysis under reduced pressure the corresponding fractions of eluate and carrying out sucrose gradient centrifugation in 0.1 M NaSCN. Due to the dilution and losses involved in the above experiments, relatively few receptor-bound counts can be brought to gradient analysis; notwithstanding these technical difficulties, repeated trials have left no doubt that, as expected, the material in the 2d peak (Stokes radius 28 Å) coincides with the 2.8S species.

Isoelectric Point. Electrofocusing on a pH 5–8 gradient of NaSCN-cytosol (0.1 M) gave the result shown in Figure 4. A single sharp peak is focused at pH 6; free estradiol-*6,7-t* is focused at about pH 7.5–8. This result was repeated. Note that the sample applied to the column contained NaSCN while the sucrose gradient was, by necessity, NaSCN free. In previous research (Puca et al., 1971), the partially purified low-salt 8.6S receptor form focused as a single peak at pH 6.2.

Inhibition of Age-Dependent Receptor Aggregation by NaSCN. The high tendency of estrogen receptor to aggregate is well known. While immediately after preparation of

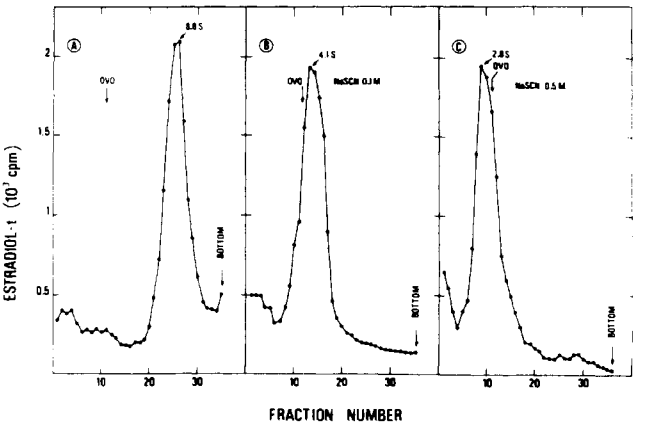

FIGURE 3: Sucrose gradient centrifugation of "low-salt" estradiol-*t*-labeled cytosol from calf uteri homogenized with TED buffer, pH 7.5. Protein concentration of cytosol was ~7 mg/ml. (A) No NaSCN. (B) NaSCN (0.1 M) present in cytosol and sucrose solution. (C) NaSCN (0.5 M) present in cytosol and sucrose solution. Sucrose gradient was 5–20%. Beckman L2-65 ultracentrifuge. Rotor: SW 65Ti. Run: 16 h at 48 000 rpm at 2 °C. Internal standard: ovoalbumin (ovo), 3.55S.

cytosol, estrogen-receptor proteins sediment at 8–9S in low salt, and at slower rates in high salt, with aging of cytosol the receptor undergoes virtually irreversible aggregation; the large aggregates rapidly sediment to the bottom of the sucrose gradient (De Sombre et al., 1969; Stancel et al., 1973a). However, if thiocyanate 0.1 M is added to cytosol, formation of the large aggregates is totally inhibited: for weeks after preparation and storage at +4 °C, receptor continues to sediment at either 4.1 or 2.8S, depending on NaSCN concentrations, and to be included in Sephadex G-200 or G-100 matrices. Also, inhibition of aggregation by NaSCN persists even during concentration procedures, like salting-out with $(NH_4)_2SO_4$, which are known to greatly favor aggregation. For best results, one must preincubate cytosol with NaSCN for at least 24 h before addition of $(NH_4)_2SO_4$.

Inhibition of aggregation is partially reversible upon removal of NaSCN by dialysis. NaSCN is virtually unable to dissociate large aggregates once formed.

Comparison of Native and Ca^{2+}-RTF Transformed Receptor Proteins in the Presence of NaSCN. Previous experiments have shown that, upon addition of Ca^{2+} (4 mM) and KCl (0.4 M) to low-salt uterine cytosol, the large 8.6S form of estrogen-receptor rapidly disappears, being replaced by a low-salt, smaller form sedimenting at 4.5S in sucrose gradient and with a 33-Å Stokes radius; the transformation is irreversible and is virtually completed within 45 min at +4 °C (Puca et al., 1972). It has been further shown that, for the transformation to occur, a separate macromolecular factor of cytosol is required, the receptor transforming factor (RTF), which is a Ca^{2+}-activated protease with high affinity for receptor; evidence suggests that transformation by RTF is probably a case of limited proteolysis (Puca et al., 1972; Bresciani et al., 1973). Differences between native and transformed estrogen-receptor forms were reinvestigated under the new dissociating conditions.

Sephadex Chromatography. Low-salt uterine cytosol (6 mg of protein/ml) was prepared and divided into two equal amounts. To one aliquot part, KCl (0.4 M) and $CaCl_2$ (4 mM) were added. After 45 min at 4 °C, NaSCN (0.1 M) was added to this "transformed" cytosol (according to the procedure described in Methods) which was then chromatographed on a calibrated Sephadex G-200 column in TED

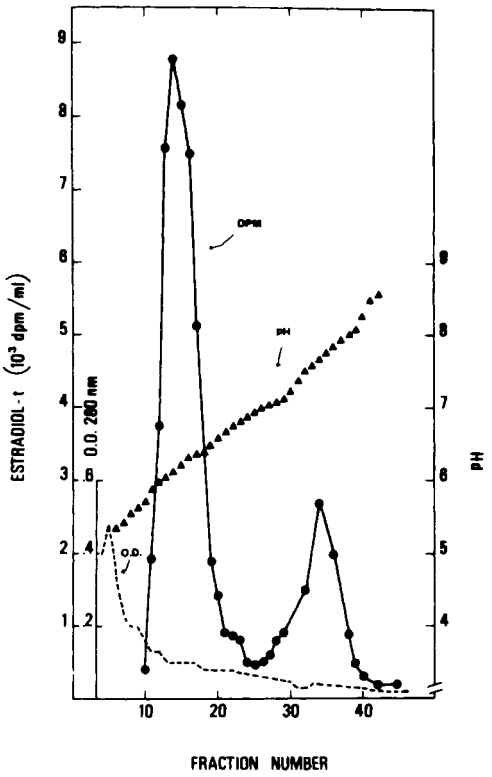

FIGURE 4: Isoelectric focusing of estradiol-*t*-binding protein in the "included" peak of Sephadex G-200 chromatography of Figure 1. By necessity, NaSCN is not present in the electrofocusing medium. Major peak (IP = 6.1) is protein bound estradiol-*t*; minor peak (IP = 7.7) is free estradiol-*t*. Electrofocusing column: standard LKB, 110 ml. Gradient: 5–50% sucrose containing 2% Ampholine pH 5–8. Sample: 20 ml of Sephadex G-200 peak (Figure 1) reduced to $^1/_{10}$ volume by dialysis under reduced pressure. Gradient was allowed to pre-focus until conductivity decreased to a constant 1.5 mA at 800 V. Selective positioning of the sample in the neutral pH range of gradient was as described elsewhere (Puca et al., 1971). Cooling water: 3 °C. Final re-focusing to 1.5 mA at 800 V. Fractions: 35 drops collected at 4 °C.

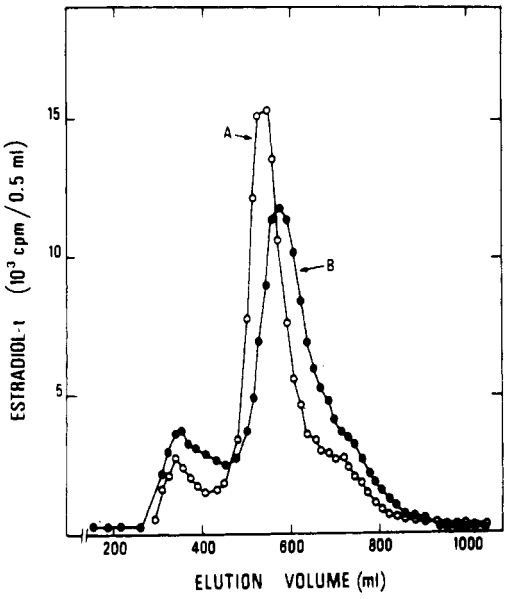

FIGURE 5: Chromatography on a calibrated Sephadex G-200 column of (A) cytosol from calf uteri homogenized in TED, pH 7.5, buffer, preincubated for 1 h at 4 °C with KCl 0.4 M; and (B) calf uterus cytosol preincubated for 1 h at 4 °C with 0.4 M KCl *and* 4 mm $CaCl_2$. After preincubation and before chromatography, both cytosols were dialyzed for 4 h against TEDSCN, pH 7.5, buffer (containing NaSCN (0.1 M)). NaSCN (0.1 M) present in both cases in column buffer (TEDSCN, pH 7.5). Relative to pattern A (peak V_e = 543 ml), pattern B (peak V_e = 581 ml) is shifted the equivalent of about −2 Å in Stokes radius. Protein concentration of cytosol was about 6 mg/ml. The column was a standard Pharmacia K50/100. Upward flow: 30 ml/h. Fractions: 7.75 ml. Calibration data: V_i = 1472 ml; blue dextran, V_0 = 341 ml; IgG, V_e = 450 ml; plasma albumin, V_e = 546 ml; chymotrypsinogen, V_e = 915 ml; myoglobin, V_e = 1139 ml.

medium containing 0.1 M NaSCN. Control cytosol, at 4 °C, was chromatographed on the same column as soon as the chromatography of the transformed cytosol was completed. The results are shown in Figure 5. In another experiment, it was control cytosol which was chromatographed first, with no change in the result. One can see from Figure 5 that native cytosol in NaSCN (0.1 M) gives the usual peak with a trailing shoulder; the transformed cytosol shows a similar pattern; however the peak is broader and there is a small but definite and highly reproducible shift of the whole pattern toward higher elution volumes. The increase in elution volume of transformed vs. native pattern was computed to correspond to a decrease in average Stokes radius of about 2 Å.

Experiments were also carried out to resolve whether the "transformation" produced by incubation of cytosol with Ca^{2+} and KCl could not be simply a consequence of Ca^{2+} binding to receptor, without any requirement for RTF. Low-salt uterine cytosol was prepared and part of it preincubated with $CaCl_2$ (4 mM) only, a procedure which is known to initiate the activation–inactivation cycle of RTF without producing receptor transformation because of lack of sufficient ionic strength (Puca et al., 1972). After 60 min of incubation at 4 °C, a time sufficient to reach virtually total inactivation of RTF, NaSCN (0.1 M) was added to both the Ca^{2+} pre-treated and the control cytosols and chromatography was carried out in sequence on a calibrated, NaSCN containing Sephadex G-200 column. No difference whatsoever was found between chromatographic patterns of native cytosol without Ca^{2+} and cytosol containing Ca^{2+} and inactive RTF. Also, no difference in elution patterns was found between partially purified, RTF-free receptor (the included peak of a thiocyanate cytosol G-200 chromatography in NaSCN (0.1 M), collected and concentrated under reduced pressure) chromatographed in TEDSCN either containing or not containing Ca^{2+} (4 mM) and KCl (data not shown).

Sedimentation Rate. No significant difference in sedimentation rate between native and transformed receptor was found (data not shown). This result is not surprising considering the small difference in the Stokes radius of the two forms in the presence of thiocyanate.

Isoelectric Point. Electrofocusing on a pH 5–8 gradient of the major peak (36 Å) from chromatography on Sephadex G-100, in the presence of NaSCN, of RTF-Ca^{2+}-transformed cytosol gave the result shown in Figure 6. There is a major peak which is focused at pH 6.4, and a definite shoulder focused at about pH 6.8. Compared with untransformed receptor isoelectrofocused under identical experimental conditions (dotted line), there is a clear shift to higher pH of the isoelectric point of transformed receptor, with definite evidence that transformation results in at least two forms differing in their electric properties. These electric changes following transformation are in agreement with results of previous studies without NaSCN (Puca et al., 1972).

DEAE-Cellulose. Further demonstration that the product(s) of RTF-Ca^{2+} transformation is less acidic compared

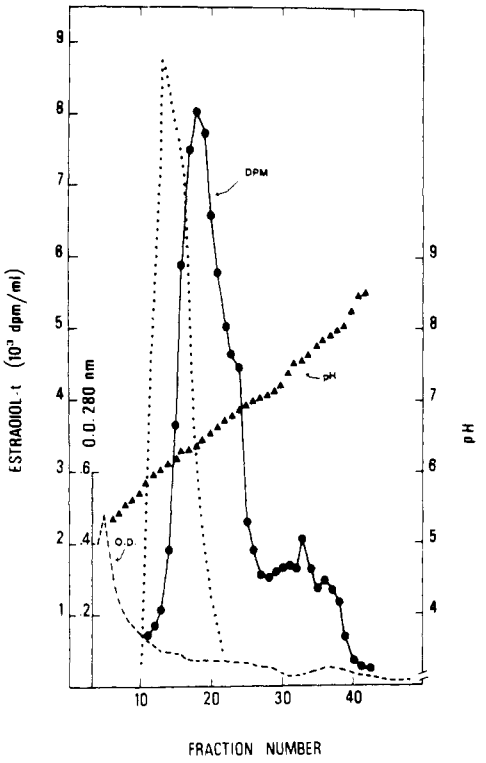

FIGURE 6: Isoelectric focusing of estradiol-*t*-binding protein in the "included" peak B of Figure 5 (●). Peak B is from "transformed" cytosol, i.e., cytosol preincubated with $CaCl_2$ (4 mM) and KCl (0.4 M) for 1 h at 4 °C, a condition which activates receptor transforming factor. Dotted line is "native" estradiol binding protein (from Figure 4). Electrofocusing conditions are as described in Figure 4.

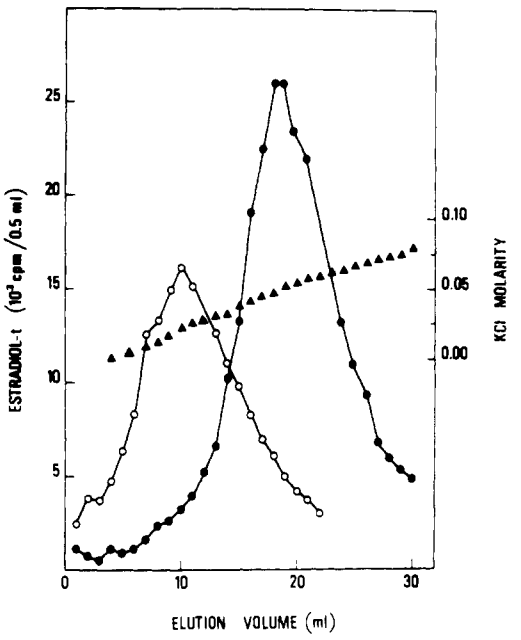

FIGURE 7: Exchange chromatography on DEAE-cellulose of estradiol-*t*-binding protein in peak A (●) or peak B (○) of Sephadex G-200 chromatographies in Figure 5. Peak A is from "native" cytosol, while peak B is from "transformed" cytosol, i.e., cytosol preincubated with Ca^{2+} (4 mM) and KCl (0.4 M), a condition which activates the receptor transforming factor. Total volume of column: 5 ml. Buffer: TEDSCN, pH 7.5, 0.05 M NaSCN. Flow rate: 30 ml/h. KCl gradient: 0–01 M. Fraction volume: 1 ml.

with native receptor is given by ion-exchange chromatography on DEAE-cellulose. From Figure 7 one can see that, after adsorption at pH 7.4 in the absence of KCl, followed by a continuous flux of buffer with linearly increasing concentration of KCl, the transformed receptor form(s) is eluted at lower KCl concentration (peak at 25 mM KCl) compared with the native form (peak at 50 mM KCl).

Discussion

Chaotropic salts impair protein–protein interaction both by direct disruption of interprotein hydrogen bonds and by disruption of hydrogen-bonded structure of water with following increase of solubility of nonpolar areas of protein surface (Sawyer and Puckridge, 1973). Contrary to urea, guanidine hydrochloride, or detergents, chaotropic salts have been shown to produce dissociation of proteins into subunits without protein denaturation (Sawyer and Puckridge, 1973). These salts, and specifically sodium thiocyanate, have now been applied successfully to block age-dependent aggregation of estrogen–receptor of calf uterus, thus removing a main obstacle to purification of this receptor in tangible amounts. Also, NaSCN favors dissociation of receptor and, furthermore, by inhibiting protein–protein interaction in cytosol, furnishes ideal conditions for the study of molecular properties of receptor in cytosol by the methods which may be applied to characterization of proteins in mixture, i.e., sucrose gradient centrifugation and exclusion chromatography on gels.

Native Receptor Forms of Cytosol in the Presence of NaSCN. Depending on NaSCN concentration and cytosol dilution, instead of the usual "8S", two estrogen-binding entities are found in low-salt calf uterus cytosol prepared using dilute Tris·HCl–EDTA buffers. These two forms may be identified from their sedimentation rate on sucrose gradients as 4.1 and 2.8S. They may be defined "native" according to a previous denomination, as opposed to "transformed" forms resulting from addition of Ca^{2+} to cytosol (Puca et al., 1972; Bresciani et al., 1973). Based on Stokes radius measurement by chromatography on calibrated columns of Sephadex G-100 and G-200, and on sedimentation coefficients obtained by sucrose gradient centrifugation, the molecular weights and frictional (and axial) ratios of the 4.1 and 2.8S entities were assessed by application of classical equations, as suggested by Siegel and Monty (1966). This method allows an unbiased estimate of molecular weights also for proteins of uncommon axial ratios and has been previously applied by us to the study of partially purified receptor preparations (Puca et al., 1971, 1972). The results of these calculations, as well as the isoelectric points from isoelectrofocusing, are presented in Table I, part A, together with the results of previous studies with partially purified receptors in the absence of NaSCN. In our view, the molecular studies with partially purified receptors are valid and fully comparable to those with NaSCN; indeed, when most of other proteins of cytosol are eliminated (more than 99%) and total protein concentration is extremely low (less than 0.01 mg/ml) as it is the case after purification, there is no reasonable expectation of significant bias in the measurement of molecular parameters of receptor by Sephadex chromatography and sucrose gradient centrifugation as a consequence of interaction of the protein of interest with other components of cytosol. Such bias is expected, and found, when the studies are with crude cytosol in the absence of NaSCN (Stancel et al., 1973a).

A Hypothesis Concerning Quaternary Structure of Native Receptor. One immediately notices from Table I, part

Table I: A Summary of Molecular Characteristics of Pre-puberal Calf Uterus Estrogen Receptor under Different Conditions.

Conditions	Stokes Radius (Å)	s	Mol Wt (Daltons)	f/f_0	Axial Ratio[c] (Prolate)	IP
			A. Native Forms			
1. Low salt, purified[a]	67	8.6	238 000	1.65	8.3	6.2
2. 0.4 M KCl, purified[a]	54	5.3	118 000	1.67	9.8	
3. Low-salt cytosol + NaSCN (0.1 M)[b]	36	4.1	61 000	1.25	3.2	6.0
4. Low-salt cytosol + NaSCN (0.5 M) *or* + NaSCN (0.1 M) and dilution[b]	28	2.8	32 000	1.44	6	
			B. Ca^{2+}-RTE Forms			
1. Low salt, purified[a]	33	4.5	61 000	1.25	3.2	6.6–6.8 (7.0)
2. Low-salt cytosol + NaSCN[b]	35	4.1	58 000	1.24	3.2	6.4–6.6
3. Low-salt cytosol + NaSCN (0.5 M) *or* + NaSCN (0.1 M) and dilution[b]	27	2.8	31 000	1.42	5.5	

[a]Molecular parameters assessed using partially purified preparation (purification factor: ×500–2500). Data from Puca et al. (1971, 1972). [b]Molecular properties do not change when KCl up to 0.4 M is added. [c]Approximate values based on chart from Oncley (1941) and assuming a hydration of 0.275 g of water per g of protein.

A, that the molecular weights of all "native" forms of receptor are nearly perfect multiples of 30 000, which is about the weight of the smallest thiocyanate species. Considering that the methods applied here furnish, however accurate, only estimates of molecular weights, the discrepancies from a perfect system of multiples may be only apparent. The simplest hypothesis to explain data in Table I, part A, is that the smallest thiocyanate form (2.8S) is a subunit which may self-associate to give dimeric (4.1S), tetrameric (5.3S), and octameric (8.6S) structures. Frictional data and strength of bonding of dimeric and tetrameric states suggest a frequent mode of association: isologous, side-by-side pairing of subunits to form a dimer and end-to-end coupling of dimers to give a tetramer, with two stronger (between subunits) and two weaker (between dimers) bonding regions. In fact, symmetry increases passing from single subunit (f/f_0 = 1.44) to dimer (f/f_0 = 1.25) and decreases passing from dimer to tetramer (f/f_0 = 1.67); also, dissociation of tetramer occurs more easily than dissociation of dimer, as expected from the above suggested model of association. Because there is an increase of symmetry, although slight, passing from tetramer to octamer (f/f_0 = 1.65), the most likely mode of tetramer interaction would be side-by-side. Of course the regions involved in intertetramer interaction must be different from those involved in dimer and tetramer formation, and this may explain why the 8.6–5.3S conversion is particularly sensitive to KCl (Erdos, 1968; Korenman and Rao, 1968; Jensen et al., 1969; Puca et al., 1972). A graphical approximation of the above hypothesis is shown in Figure 8. This basic hypothesis is compatible with more complex possibilities of receptor structures. For instance, the subunits may be *similar* but not *identical,* like in the case of hemoglobin; also, not every subunit needs to be endowed with an estrogen-receptor site.

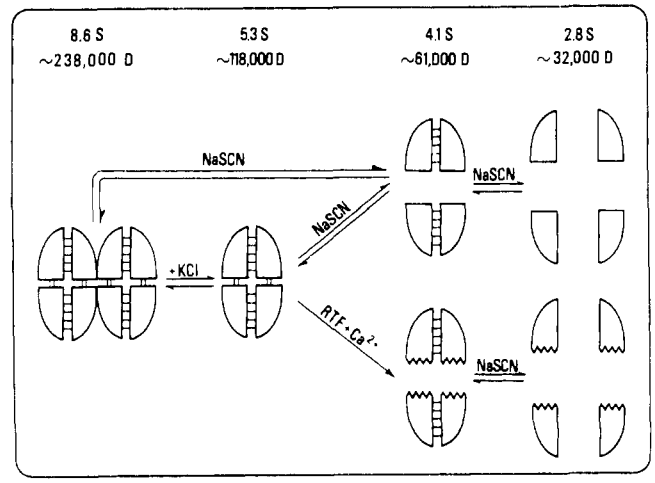

FIGURE 8: Graphical approximation of hypothesis concerning quaternary structure of receptor. The model is based on molecular weights and frictional ratios of various forms. Subunits about 30 000 daltons in weight are the basic building blocks. The hypothesis is compatible with similar, and not identical, subunits contributing to quaternary structure. NaSCN reversibly dissociates larger forms by direct impairment of intersubunit bonds. Ca^{2+}-activated receptor transforming factor, by preferential proteolytic attack of weaker bonding regions (between dimers), indirectly destroys involved bonds and irreversibly releases slightly modified, about 60 000-dalton dimers; RTF dimers may be further dissociated into subunits by NaSCN. We conclude that in vivo the effect of RTF could be instrumental in conferring mobility to cytoplasmic receptor, thus allowing nuclear penetration of the estrogen-receptor complex. See text for further discussion.

Self-Association vs. Heteroassociation of Receptor Proteins. Our data may thus be interpreted to suggest the existence of definite receptor structures of increasing complexity resulting from self-association of identical, or similar, subunits. As discussed previously (Puca et al., 1972), at ionic concentrations and protein concentrations closest to values of uterine cell, the higher states of association (tetrameric or higher structures of present hypothesis) are those more likely to be in existence. Self-association, it must be pointed out, does not, however, exclude heteroassociation of receptor states with other macromolecules of cytosol. Heteroassociation may be nonspecific, resulting from artificial conditions following cell disruption, and the age-dependent formation of large aggregates in uterine cytosol appears to be, beyond reasonable doubts, a consequence of such type of heteroaggregation, or have a physiological significance, as

suggested by Jensen and De Sombre (1973) in the case of the temperature dependent "4S" to "5S" transformation; the temperature dependent "5S" is thus distinct from the native 5.3S described here and in previous papers (Puca et al., 1971, 1972), and which is found in nonheated cytosol.

Receptor Forms in Cytosol after Addition of Ca^{2+} and KCl. When calf uterus cytosol is preincubated with Ca^{2+} (4 mM) and KCl (0.4 M), sucrose gradient centrifugation and Sephadex chromatography in the presence of NaSCN give results similar but not identical with those obtained with "native" cytosol. The 4.1S and a 2.8S estrogen-binding entities are always found but they show some slight but definite differences from the corresponding native entities: slightly smaller apparent Stokes radii and molecular weights, and decreased homogeneity as shown by broader peaks on chromatography and a multispiked band shifted toward lower pH on electrofocusing; also, there is no reversion of 4.1S to faster sedimenting states after removal of NaSCN. Molecular data for these forms are summarized in Table I, part B, which includes calculations of molecular weights and frictional ratios by the same method applied to native forms.

Proteolytic Processing of Receptor by RTF. Previous research (Puca et al., 1972; Bresciani et al., 1973) has shown that Ca^{2+} acts via activation of a separate factor of cytosol, the receptor transforming factor, which is a Ca^{2+}-activated proteolytic enzyme with high affinity for the 118 000-dalton receptor forms. When added to cytosol containing 0.2–0.4 M KCl, Ca^{2+} starts a rapid activation–inactivation cycle of RTF, producing a flash of proteolytic activity and irreversible formation of an about 60 000-dalton receptor species.

We suggest the following interpretation of present and past data. RTF, by preferential proteolytic attack of the weaker bonding region of tetramer, indirectly destroys the involved bonds and thus releases somewhat modified dimers (effect irreversible). NaSCN (0.1 M) similarly dissociates the tetramer into dimers, however, by direct disruption of the involved bonds which may thus re-form after removal of NaSCN (effect reversible). Previous (Puca et al., 1972) and present experiments exclude the possibility that, under our conditions, the effect of Ca^{2+} is a direct consequence of conformational modifications following binding of Ca^{2+} to receptor.

The ability of NaSCN and proteolytic enzymes to produce similar effects on quaternary structure of proteins is not new. For instance, both, trypsin and NaSCN dissociate rabbit skeletal muscle protein kinase into a cAMP-regulatory subunit and an active catalytic subunit (Huang and Huang, 1975). In this case, too, the effect of NaSCN is reversible while that of the protease is irreversible and the explanation is the same: NaSCN impairs intersubunit bonds directly, while the enzymes act via proteolysis of bonding regions, with indirect disruption of intersubunit bonds.

Subunit Studies by Others. Erdos and Fries (1974) have studied the subunit structure of estrogen–receptor by the technique of gel filtration in denaturing solvents, specifically 6 M guanidine hydrochloride. After filtration the receptor could be partially renatured and detected by its estrogen-binding ability. They conclude that the subunit of native receptor is 55 000 daltons in weight, while the RTF-treated receptor is 35 000 daltons in weight. Thus, their results for the RTF subunit agree quite well with ours. The lack of detection by these authors of a similar small subunit for the *native* receptor may have the following explanation: they probably achieved only incomplete dissociation and their conditions of gel filtration did not allow for complete separation of subunit and dimer; indeed, on Sephadex G-200 their major peak (55 000 daltons units) shows a shoulder which may be, like in our Sephadex G-200 chromatography of Figure 1, the unresolved small "native" subunit.

Also, a progesterone-binding subunit sedimenting at 2.6S and with an apparent molecular weight of 20 000 daltons has been found for the progesterone receptor of chick oviduct by Sherman et al. (1974). This subunit is formed in the presence of several divalent cations, with an order of effectiveness ($Ca^{2+} > Mn^{2+} > Sr^{2+} > Ba^{2+} > Mg^{2+}$ ineffective) which is the same as found for activation of RTF in calf uterus cytosol (Puca et al., 1972).

Ca^{2+}, Proteolytic Processing of Receptor and Initiation of Estrogen Action. As suggested earlier (Puca et al., 1972; Bresciani et al., 1973), proteolytic processing of receptor may be involved in the cytoplasm-to-nucleus transfer of estrogen–receptor complex in the uterine cell. RTF is not a lysosomal protease as shown, among other properties, by its optimum of activity at pH 8.7, and shows a rapid activation–inactivation cycle following increase of Ca^{2+} concentration to 4 mM. The RTF product, furthermore, has irreversibly lost most of the tendency to aggregate typical of the native form. One may thus envisage that early changes of Ca^{2+} levels in the uterine cell following estrogen penetration of uterus may activate RTF which in turn mobilizes receptor previously confined in the cytoplasm by "loosening" 60 000-dalton forms from larger, immobile superstructures, by indirect perturbation of weak interactions via limited proteolytic attack of specific bonding regions in the superstructures. This hypothesis is consistent with the data showing very early "permeability" changes following estrogen interaction with the uterine cell (see Riggs, 1970) and, specifically, with the finding by Petras and Szego (1975) that physiological levels of estrogen influence rates of Ca^{2+} exchange as early as 2.5 min after in vitro addition of hormones to suspension of endometrial cells from uteri of ovariectomized rats.

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Effect of Bridging the Two Essential Thiols of Myosin on Its Spectral and Actin-Binding Properties†

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ABSTRACT: The circular dichroic and fluorescent spectral properties of the myosin head (subfragment I (SFI)) modified by covalently bridging the two essential thiol groups have been examined. CD spectra of SFI with the two thiols linked through reaction with a bifunctional reagent, *N,N'-p*-phenylenedimaleimide, show enhancement of the 282-nm minimum similar to that observed for the long-lived kinetic intermediate (Mg**MgADP·P_i) formed during the ATP cleavage reaction. No significant perturbation of the CD band at 282 nm is seen on blocking both thiol groups with the monofunctional reagent *N*-ethylmaleimide. The fluorescence emission maximum also shifts to lower wavelengths following covalent bridging (from 343 to 340 nm), but no change in fluorescent intensity has been detected. Formation of the covalent bridge completely inhibits interaction of the modified protein with F-actin. These results suggest that the local conformational state of the polypeptide chain formed on bridging the two thiol groups exhibits certain similarities with the state produced following binding of MgATP to native myosin.

Recent stopped-flow kinetic studies have provided a detailed reaction sequence for cleavage of MgATP by myosin. The reaction takes place in a series of steps (at least seven) which lead ultimately to release of P_i and MgADP. These different states have been characterized by a variety of optical techniques employing rapid and steady-state analyses by fluorescence (Werber et al., 1972; Mandelkow and Mandelkow, 1973a,b; Bagshaw et al., 1974), uv difference spectroscopy (Morita, 1967), circular dichroism (Murphy, 1974), and ESR spectroscopy (Seidel and Gergely, 1973; Stone, 1973). A primary feature of the MgATP–myosin interaction is the existence of a relatively long-lived intermediate, designated M**MgADP·P_i (Bagshaw et al., 1974), which is responsible for the very low turnover rate of the substrate. The conformational state M**MgADP·P_i is observed only in the presence of MgATP and is not detected when the products of hydrolysis, P_i and MgADP, or nonhydrolyzable analogues of the substrate, e.g., MgAMP-PNP or MgAMPPCP, are mixed with myosin. To account for the unique character of this conformation and for the slow rate of its decay to the subsequent products, it has been proposed that MgATP forms a stable cyclic ternary complex with the two essential thiol sites within each head of myosin, SH_1 and SH_2 (Burke et al., 1973; Reisler et al., 1974a).

If formation of the unique conformation of the M**MgADP·P_i state involves a structural transition which brings the SH_1 and SH_2 groups into close steric proximity, and if these two groups could be covalently "trapped" in this conformation, it may be reasoned then, that the optical properties of the polypeptide chain in this state might resemble the long-lived intermediate even in the *absence* of the substrate.

In the study to be described below, we have determined the circular dichroism and fluorescence spectra of subfragment I modified by covalently linking the two essential thiols with the bifunctional reagent *N,N'-p*-phenylenedimaleimide. Our experiments indicate that the CD properties of the covalently formed ring structure and the

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