Characterization of the Estradiol Receptor Extracted from Nuclei by Micrococcal Nuclease†

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ABSTRACT: The estradiol receptor of lamb endometrium, translocated to nuclei in utero and extracted with micrococcal nuclease at 2–4 °C, was compared under low salt conditions to the "native" 8S and to partially proteolyzed cytosol receptor. The degree of extraction of the receptor was roughly parallel to the proportion of digested chromatin and to the yield of extracted DNA. After extensive digestion of chromatin, which produced up to 10% perchloric acid soluble DNA and more nucleosome monomer than dimer, up to 80% of the nuclear receptor was extracted under low ionic strength and subsequently analyzed by ultracentrifugation and chromatography. This nuclear receptor appeared heterogenous. It migrated with sedimentation constants of 4 and 6 S. The Stokes radius of the predominant form was 5.3 nm. The calculated apparent molecular weights were 130 000 and 90 000, respectively. The nuclear receptor was eluted from the diethylaminoethylcellulose column at 0.23 and 0.30 M KCl, and both forms of the receptor were able to bind DNA. Finally, the nuclear receptor was reversibly dissociated by 0.5 M KCl into 3.8S components of 45 000 daltons. We conclude that the majority of the nuclear estradiol receptor extracted under mild conditions by micrococcal nuclease is different from the partially proteolyzed cytosol receptors obtained by the Ca^{2+}-transforming factor and therefore does not derive from any of these forms but rather from the native 8S receptor. In addition, the nuclear receptor appeared to bind more weakly to nucleosomes than to naked DNA, as its interaction with slightly digested chromatin was completely dissociated by double-stranded DNA.

The action of estrogen in their target cells is mediated by specific soluble receptors (R)[1] which are found either in the nucleus or in the cytosol according to the presence or absence of estrogens (Gorski and Gannon, 1976; Jensen and De Sombre, 1972). The estrogen-dependent formation of the nuclear receptor is one of the first steps which follow hormone binding and is probably involved with hormonal stimulation of RNA synthesis in the nucleus. Consequently, it is important to understand its mechanism of formation and to specify the relationship between the cytosol and nuclear receptors (Rochefort, 1970).

It is now generally accepted that the nuclear receptor (Rn) derives directly from the estradiol (E_2)-cytosol receptor (Rc) complex and that the hormone is not transferred from the cytosol receptor to another protein localized in the nucleus. However, the nature of the activation of the RcE_2 complex which leads to its nuclear translocation through the nuclear membrane is unknown. The comparison of the physicochemical properties (and mostly of the molecular weight) of the nuclear and cytosol receptors leads to three series of hypotheses: (1) The RcE_2 complex undergoes a conformational change without modification of the molecular weight. (2) The RcE_2 complex is transformed into a heavier form by dimerization or interaction with other components (Notides and Nielsen, 1974). (3) Finally, the RcE_2 complex is transformed into a smaller receptor either by reversible dissociation of Rc into subunits or by an irreversible proteolysis, in order to pass through the pores of the nuclear membrane. The Rc proteolysis was suggested by Bresciani et al. (1973) and by Puca et al. (1977), who demonstrated the presence of a Ca^{2+}-activated transforming factor which catalyzes a partial proteolysis of the cytosol receptor into a 4S Ca^{2+} Rc. These authors could not find any difference between this Ca^{2+} Rc and the purified nuclear R (Puca et al., 1970). Rc was also shown to be split by an endogenous serine protease (Notides et al., 1974). The contradictory results that have been reported (Gorski and Gannon, 1976) concerning the relationship between the cytosol and nuclear receptors may be due to two kinds of artifacts. The nuclear receptor has up to now been mainly obtained after extraction by high ionic strength (KCl $\geq$ 0.3 M) which modifies the interaction of the cytosol receptor with other proteins or between subunits (Rochefort and Baulieu, 1971; Erdos and Fries, 1974; Korenman and Rao, 1968), the subsequent removal of salt leading to aggregations. In addition, every prolonged analytical procedure can favor proteolysis of the receptor (Rochefort and Baulieu, 1972). We therefore tried to prepare the nuclear receptor under milder conditions so as to avoid or minimize artifactual aggregation and proteolysis. The nuclear translocation was obtained in a surviving uteri system which mimics the physiological situation better than does the cell-free system (Rochefort et al., 1972) and which gives a higher yield of nuclear receptor (unpublished). The nuclear R was then extracted and analyzed in a low salt medium after treatment of nuclei with the micrococcal nuclease which solubilizes chromatin and liberates nucleosomes. The experimental conditions were adapted in order to minimize artifactual proteolysis of the receptor.

The present paper reports evidence that the nuclear receptor of estradiol, extracted from purified nuclei after digestion by micrococcal nuclease, is different from the partially proteolyzed receptors obtained from the cytosol but similar to the

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[1] Abbreviations used are: R, estradiol receptor; Rn, nuclear estradiol receptor; Rc, cytosol estradiol receptor; Ca^{2+}Rc, Rc partially proteolyzed by a Ca^{2+}-transforming factor; E_2, 3,17β-dihydroxy-1,3,5(10)-estratriene; TS, 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose; TSM, TS with 3 mM $MgCl_2$; TS Ca, TS with 1 mM $CaCl_2$; TE, 10 mM Tris-HCl (pH 7.4), 1.5 mM EDTA; TEK, TE with 0.5 M KCl; TET, TE with 12 mM thioglycerol; DCC, dextran-coated charcoal; PCA, perchloric acid; NE, nuclear extract; micrococcal nuclease Rn, the micrococcal nuclease extracted nuclear receptor; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; EDTA, (ethylenedinitrilo)tetraacetic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

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native 8S cytosol receptor. These results indicate Rc is not partially proteolyzed in vivo during its nuclear translocation.

Materials and Methods

Uteri from immature lambs, weighing less than 6 g, were used within 60 min after slaughter. Except when stated, all steps were performed at 2–4 °C.

Preparation of Rc-Containing Extracts. Cytosol (2–4 mg of protein/mL) was prepared from the total uterus in 10 mM Tris-HCl (pH 7.4), 1.5 mM EDTA buffer (TE) as described previously (Rochefort and Baulieu, 1971). It was then incubated with 3–10 nM tritiated estradiol (specific activity 60 Ci/mmol) for 90 min at 2 °C to allow the formation of the RcE_2 complex. Cytosol treatment by trypsin (TPCK, Worthington) was performed at 2 °C for 30 min at 10 μg/mg of protein (Erdos and Fries, 1974) in order to produce the trypsin Rc. To obtain the Ca^{2+} Rc, the RcE_2 complex was partially proteolyzed by a Ca^{2+}-transforming factor according to Puca et al. (1977) and purified three- to fivefold by ammonium sulfate precipitation. The extract was then desalted on a Sephadex G-25 column equilibrated in TE buffer. Similar transformation was obtained from lamb endometrium.

Preparation of RnE_2-Containing Extracts. Whole uteri, open lengthwise, were incubated for 1 h at 37 °C in Eagle's medium. One horn was incubated with $^3HE_2$ (20 nM) and the other horn was incubated with $^3HE_2$ (20 nM) and 2 μM of nonradioactive E_2 for nonspecific uptake. The endometrium was scraped off and their nuclei were purified with a 40% yield according to a slight modification of Allfrey et al. (1964), in a 10 nM Tris-HCl (pH 7.4), 0.25 M sucrose buffer (TS) containing 3 mM $MgCl_2$ (TSM). They were 90% pure as checked by light and electron microscope examination, and their weight proportions for proteins, DNA, and RNA were 3.1 and 0.4, respectively.

All buffers used for preparing and digesting nuclei contained phenylmethanesulfonyl fluoride (0.2 mM) to minimize proteolysis. The nuclei were then extracted according to three different procedures:

(1) Enzyme Extraction. The purified nuclei were washed and resuspended in TS containing 1 mM $CaCl_2$ (Ts Ca).

The concentration of nuclei per milliliter was evaluated in Thoma's cell by counting more than 600 nuclei in duplicate, and the suspension was adjusted to $1–3 \times 10^8$ nuclei/mL (0.7–2.1 mg of DNA) and stirred for 1 h at 2–4 °C with micrococcal nuclease (Worthington (EC 3.1.4.7) at 750 units/mg of DNA. The reaction was stopped by adding 10 mM EDTA. The suspension was centrifuged for 20 min at 600*g*, and the nuclear extract (NE_1) was separated from the pellet (P_1) which was then suspended in TE buffer for 30 min in order to lyse the nuclei. This suspension was centrifuged for 20 min at 800*g*, and the nuclear extract (NE_2) was separated from the pellet (P_2). The total extracted material ($^3HE_2$ or DNA) was obtained by combining the two extracts.

(2) Sonication. The purified nuclei were washed and resuspended in 1 mL of TE buffer for 30 min. They were then sonicated with a microtip in a MSE sonicator for 1–4 min in 15-s pulses at medium power, amplitude 4 μm, followed by 1-min cooling intervals between pulses in a salted ice bath at −10 °C. The temperature of the suspension measured immediately after sonication was 10–12 °C. The nuclear extract was obtained after centrifugation at 600*g* for 20 min.

(3) TE Extraction with or without KCl. The nuclei contained in TSM buffer were centrifuged at 600*g* for 20 min and subsequently resuspended either in 1 mL of TE buffer or 1 mL of TE buffer containing 0.5 M KCl and incubated 30 min at 2 °C. The suspension was then centrifuged at 600 *g* for 20 min, and the supernatants were designated as the TE or the KCl nuclear extract.

Sucrose Gradient Ultracentrifugation. The samples were previously incubated with a suspension of 0.05% dextran, 0.5% charcoal Norit A (DCC) in TE buffer for 5 min in order to adsorb the free estradiol. In addition, the sucrose concentration of NE_1 was diluted twofold. Sedimentation coefficients were determined according to Martin and Ames (1961) by using either endogenous hemoglobin (4.2 S) or bovine Ig globulin (7 S) and ovalbumin (3.8 S) as internal standards. The monomer subunit of the chromatin (11 S) was also used as an internal standard for nuclear receptor. The absorbancy at 260 nm, or at 410 nm for hemoglobin, and the radioactivity were measured in each fraction after collection through the bottom of the tube. The DCC was pelleted and the supernatants were then layered on an isokinetic 5–20% sucrose gradient prepared in TE buffer. The gradient was exponential and prepared according to Noll (1967) when the SW_{41} rotor was used; it was linear when the SW_{50} rotor was used.

Gel Filtration. Ultrogel ACA 34, LKB, or Sepharose 6B, Pharmacia, was degassed, packed, and equilibrated with TE buffer before use. The filtration flow rate was 15–25 mL/h. The distribution coefficient K_d was calculated as indicated in the legend of the Figure 3. The Stoke radii (R_S) of the different forms of receptors were determined from duplicate experiments according to Porath (1963). The gel-filtration column was standardized using sperm whale myoglobin (R_S 2.02), ovalbumin (R_S 2.8), bovine serum albumin (BSA) (R_S 3.63), BSA dimer (R_S 4.2), bovine liver catalase (R_S 5.22), horse apoferritin (R_S 6.14). When samples had been prepared in TEK buffer, the column was equilibrated and calibrated with the corresponding buffer.

DEAE-cellulose Ion-Exchange Chromatography. DEAE-cellulose chromatography was performed according to Schrader and O'Malley (1972). Microgranular DEAE-cellulose (Whatman DE-52) was extensively washed with TE buffer containing thioglycerol (12 mM) (TET) and packed in a 10-mL column. After addition of the cytosol or nuclear extracts, the column was washed with 40 mL of TET buffer and the elution was carried out with a linear 112-mL KCl gradient (0–0.5 M KCl) in TET; 1.5-mL fractions were collected, and aliquots were assayed for radioactivity and optical density. KCl molarity was evaluated from the refractive index measured with Abbe's refractometer VEB Carl Zeiss JENA DDR. The samples containing 0.5 M KCl were desalted on a Sephadex G-25 column.

Measure of the Estradiol–Receptor Complexes. The E_2R complexes bound to nuclear pellet before or after extraction were evaluated by the difference between the radioactivity retained by nuclei prepared from uterus incubated with $[^3H]E_2$ alone and that prepared from uterus incubated with $[^3H]E_2$ and a 100-fold excess of nonradioactive E_2. The E_2R complexes contained in the nuclear extracts were measured by the dextran charcoal assay (Rochefort and Baulieu, 1972). In this case, the nonspecific binding was the $[^3H]E_2$ binding in nuclear extract prepared from uterus incubated with $[^3H]E_2$ and an excess (2 μM) of nonradioactive E_2. When indicated, the specific binding was also evaluated by protamine sulfate precipitation (0.5 mg/mL) for 15 min at 2 °C according to Chamness and McGuire (1975).

Miscellaneous. Radioactivity was measured in 3 mL of ethanol–10 mL of toluene POPOP–PPO scintillating mixture with a constant efficiency of 20% evaluated by external standard. The proteins were measured according to Hartree (1972) or by absorption at 280 and 260 nm (Warburg and Christian,

TABLE I: Repartition of $^3HE_2$ and DNA in Nuclei and Low Salt Nuclear Extract.[a]

mode of extract.	expt. no.	E_2 bind.[b] sites/ nucleus	% $^3HE_2$ in nuclear extracts: total[c] $^3HE_2$	total[c] +cold	bound[d] $^3HE_2$	bound[d] +cold	% DNA in[b] nucl extr
micrococcal nuclease ($NE_1 + NE_2$)	1	13 800	55.7	4.1	43.7	0.5	88.6
	2	28 100	66	8.0	53.5	8.0	55.7
	3	22 100	89		65.5		
	4	17 200	86		50.9		74
	5	38 400	90	8.9	72.9	1.6	
	6	51 800	80	1.3	68.8	0.7	
	7	20 700	80				
	8	16 300	76		72.8		
	9	50 000	55		38		61.5
	mean ± SD	28 700 ± 14 500	75.3 ± 13.4	5.6 ± 3.5	58 ± 13.5		70 ± 14.6
sonication	11	13 100	77				75.9
	12	27 700	87.8		15.1		86.6
	13	32 800	82.5		27.4		88.0
	14	26 700	88.7		17.5		87.9
	mean ± SD	25 100 ± 8400	84 ± 5.4				84.6 ± 5.8
lysis	15	13 100	3.2				5

[a] Lamb uteri were incubated for 1 h at 37 °C in Eagle's medium with [^{3}H]E_2 (20 nM) ± nonradioactive E_2 (2 μM). The endometrial nuclei were purified and then extracted after micrococcal nuclease digestion or by sonication in TE buffer or by lysis in TE buffer as described under Materials and Methods. [b] The number of E_2 binding sites per nucleus was calculated according to their saturable uptake of [^{3}H]E_2 based on a DNA content of 7.7 pg/endometrial cell. The nonsaturable uptake of [^{3}H]E_2 in nuclei obtained with an excess of nonradioactive E_2 represented between 3 and 14% of that incorporated without isotopic dilution. [c] The percentages of [^{3}H]E_2 and of DNA totally recovered in the nuclear extracts are represented in taking as the 100% value for each experiment the total DNA and the [^{3}H]E_2 incorporated into nuclei in the absence of nonradioactive E_2. [d] The bound [^{3}H]E_2 was measured in the two nuclear extracts ($NE_1 + NE_2$) by the DCC assay for 16 h at 2 °C as described under Materials and Methods.

1941). Calf thymus DNA was prepared according to Marmur (1961) and used directly or after sonication. DNA was measured in the material precipitated by and washed in 5% PCA final concentration according to Burton (1968) using calf thymus DNA as a standard. The PCA-soluble DNA was evaluated by OD at 260 nm (1 OD_{260} unit = 37.5 μg/mL). RNA was evaluated according to Schneider (1957) with yeast RNA used as a standard.

Results

Nuclear Translocation and Extractions of the Estradiol Receptor. After incubation of lamb uteri in Eagle's medium for 1 h at 37 °C with a physiological concentration of [^{3}H]E_2 (20 nM) between 13 100 and 51 800, E_2 binding sites were found per endometrium nucleus (Table I). The reasons for this variability have not been determined. It could be due to the different hormonal status of the animals and/or the different delay between the sacrifice of the animals and the incubation of uteri. Ninety percent of this nuclear uptake was prevented by a 100-fold excess of nonradioactive estradiol. Conversely, less than 2000 molecules were specifically bound by isolated nuclei after their cell-free incubation with [^{3}H]E_2 in the absence of Rc (results not shown). This result indicated that a small proportion of the accessible or easily exchangeable estradiol binding sites was located in the nuclei before uterus exposure to estradiol.

When purified nuclei from labeled uteri were treated with high concentrations of micrococcal nuclease and then lysed, a mean of 75% of the nuclear [^{3}H]E_2 was solubilized and 58% was bound to macromolecules, as assayed by dextran-coated charcoal adsorption (Table I). This binding (90–99%) disappeared after heating for 10 min at 60 °C, treatment by 1 mg/mL Pronase for 10 min at 37 °C, or precipitation by protamine sulfate, thus strongly suggesting that this binding was due to the RnE_2 complex. Moreover, the concentrations of the DCC-resistant [^{3}H]E_2 binding sites contained in the nuclear extracts from uteri incubated with [^{3}H]E_2 in the presence or absence of nonradioactive estradiol were not modified by a further addition of 3 nM [^{3}H]E_2. Consequently, the low level of [^{3}H]E_2 DCC resistant binding recovered in nuclear extracts from uteri incubated with [^{3}H]E_2 and an excess of nonradioactive E_2 was not due to the small amount of radioactivity incorporated and was therefore an adequate way of estimating the nonspecific binding. The nuclear extracts prepared from the uterus incubated in the absence of E_2 and subsequently incubated with [^{3}H]E_2 contained less than 2% of the [^{3}H]E_2 DCC resistant binding sites, confirming that the majority of the nuclear receptors required the presence of estradiol.

The solubilization of the [^{3}H]E_2 by micrococcal nuclease was roughly parallel to that of DNA (Table I). In most cases, the two nuclear extracts contained 60 to 80% of the total DNA in the PCA-precipitable fraction and a maximum of 10% in the PCA-soluble fraction, the remaining DNA being pelleted. The repartition of the estradiol receptor between the two nuclear extracts NE_1 and NE_2, obtained after enzyme treatment and subsequent lysis of nuclei, varied markedly from one experiment to another, since the ratio of the bound [^{3}H]E_2 in NE_1 over that in NE_2 varied between 0.06 and 6.70.

After sonication of nuclei from [^{3}H]E_2-labeled uteri, a mean of 84% of [^{3}H]E_2 was extracted, but only 20% was bound to R as shown by DCC assay, suggesting that this procedure altered the Rn. The amount of [^{3}H]E_2 extracted from nuclei was also parallel to that of the solubilized DNA.

After lysis of nuclei in TE buffer, only 3% of [^{3}H]E_2 and DNA was extracted, thus confirming that the large majority of the Rn remained bound to the nuclear pellet after centrifugation in TE buffer (Shyamala and Gorski, 1969).

These results indicate that the physical or enzymatic shearing of chromatin and not only the opening of the nuclear membrane is needed to extract the nuclear receptor in a salt-free medium.

Sucrose Gradient Analysis of the Nuclear Extracts. In order to determine whether the extracted nuclear receptors

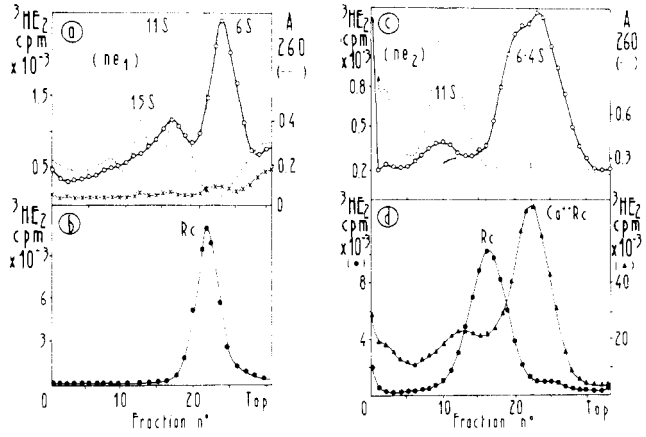

FIGURE 1: Ultracentrifugation in a sucrose gradient. The two nuclear extracts (a and c) and the cytosol extracts containing either the native cytosol receptor (Rc, ●) or the partially proteolyzed receptor (Ca^{2+}Rc, ▲) were prepared as indicated under Materials and Methods. All extracts were treated by DCC for 5 min at 2 °C and then ultracentrifuged on an isokinetic 5–20% sucrose gradient at 38 000 rpm for 14 h at 0.2 °C, using an SW 41 (a,b) or an SW 50 (c,d) rotor. The absorbance at 260 nm (- - -) and the radioactivity (—) were measured in each fraction. The *s* value was determined according to internal standards as described under Materials and Methods. (a) Nuclear extract NE_1 containing 54 and 67.5% of the nuclear E_2R and DNA, respectively. The nonspecific interaction of $^3HE_2$ (X) was obtained by isotopic dilution during the incubation of uteri. (b) Cytosol containing the 8S receptor prepared from the same uteri as a and analyzed in parallel. (c) Nuclear extract NE_2 prepared in a different experiment as in a. It contained 62 and 56% of the nuclear E_2R and DNA, respectively. (d) Ca^{2+}Rc and 8S Rc prepared from the same uteri as c and analyzed in parallel. The same nuclear extracts (NE_2) or Ca^{2+}Rc-containing extracts were analyzed in parallel by gel filtration (Figure 3c,d).

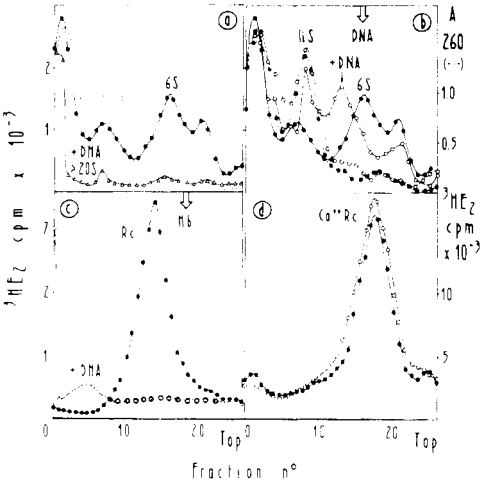

FIGURE 2: DNA binding ability of the different molecular species of the receptor. The following extracts were prepared as described under Materials and Methods and in Figure 1. (a,b) Micrococcal nuclear extract (NE_2) from endometrium nuclei purified from $[^3H]E_2$-labeled uteri. The extracted E_2R and DNA represented 38 and 61% of the total nuclear E_2R and DNA, respectively. (c) Uterine cytosol incubated with 2 nM $[^3H]E_2$. Hb = hemoglobin as internal standard. (d) Partially proteolyzed and purified Ca^{2+}Rc. The extracts were then treated by DCC for 5 min at 2 °C and subsequently incubated with (○) or without (●) calf thymus DNA (final concentration 200 μg/mL). We used either heavy DNA (>20S) a, c, d) or sonicated 6S DNA (b). After 60 min of incubation at 2 °C, 0.5-mL aliquots were centrifuged in a linear 5–20% sucrose gradient at 39 000 rpm for 18 h in an SW 41 rotor. The chromatin material was followed by the absorbance at 260 nm (- - -) and the E_2-receptor complexes by the radioactivity (—).

were bound to chromatin or were free, we compared the radioactivity and the absorbance patterns of these nuclear extracts after ultracentrifugation. We defined as free the receptors labeled by $[^3H]E_2$ which migrated slower than the mononucleosome peak (11 S), even though we could not exclude the possibility of an interaction of the receptor with other proteins or nucleic acids. After sonication of the nuclei, the majority of the DCC-resistant $[^3H]E_2$ comigrated with the material absorbing at 260 nm, without any enrichment of $[^3H]E_2$ per A_{260} unit. A maximum of 5% of RnE_2 appeared to be free in the 4S region of the gradient (results not shown). It was most likely that E_2, with possibly its receptor, interacted with chromatin under these conditions, since the sonication procedure did not result in aggregation of Rc. However, since it is well known that sonication induces a redistribution of nuclear proteins, we did not further characterize the receptors obtained after sonication.

After micrococcal nuclease digestion, the 260-nm absorbance pattern (Figure 1) showed three peaks at about 11, 15, and 19 S corresponding to the *s* values of the mono-, di-, and trimers of the chromatin subunit, as described in other tissues (Noll, 1974). A similar pattern was shown when the DNA concentration in each fraction was measured by Burton's technique. In six experiments where the amount of monomers was predominant, 60 ± 10% (mean ± SD) of the layered $[^3H]E_2$ was bound to 4S and 6S peaks and did not comigrate with chromatin. This free RnE_2 complex represented from 24 to 45% of the intranuclear receptor. In two other experiments in which the chromatin was digested less extensively, only 14 and 26% of $[^3H]E_2$ was liberated in the 4–6S region. These results suggested that the degree of liberation of the receptor from chromatin was related to the extent of chromatin digestion.

As the aim of this paper is to characterize the solubilized Rn, we have mainly reported the results obtained after extensive digestion of the chromatin. The pattern observed was generally different in the first (NE_1) and the second (NE_2) nuclear extracts. The first extract contained diffusible material consisting of chromatin subunits generally smaller than 30S. In the second extract obtained after lysis of nuclei, the proportion of the heavier chromatin was higher. The large majority of the radioactivity migrated as a 6S peak (Figure 1a) or as two entities 6 and 4 S (Figure 1c), in the first and second nuclear extract, respectively. The significance of this heterogeneity remains to be determined. The 6 and 4S peaks were depleted when the uterus was labeled in the presence of a 100-fold excess of nonradioactive E_2 (Figure 1a). In addition, they were not found in nuclear extracts prepared from unlabeled uteri which were subsequently incubated with 3 nM $[^3H]E_2$ for 1 h at 2 °C and treated by DCC for 10 min (not shown). We therefore concluded that the $[^3H]E_2$ 4–6S peaks represented the estradiol-nuclear receptor either free or interacting with nondefined molecules. The sedimentation constant of the $[^3H]E_2R$ complex was not significantly different following partial proteolysis by a Ca^{2+}-activated factor (4–5 S) but was always slower than that of the 8S "native" receptor (Figure 1b–d). Since the cytosol and nuclear receptors were not purified, their different rate of migration could be due to the receptor itself or to other components of the extracts as has been suggested (Stancel et al., 1973). However, the protein concentration, which was similar in both extracts, could not serve as a simple explanation for the different sedimentation constants. We have verified that the treatment of the cytosol with micrococcal nuclease did not modify the sedimentation of the 8S R. Conversely, the 6S Rn could not be transformed into 8S by treatment of NE_2 with cytosol containing nonradioactive E_2 to occupy the R sites.

A small peak of radioactivity localized in the 10–12S region was occasionally observed (Figure 1a–c); however, its signif-

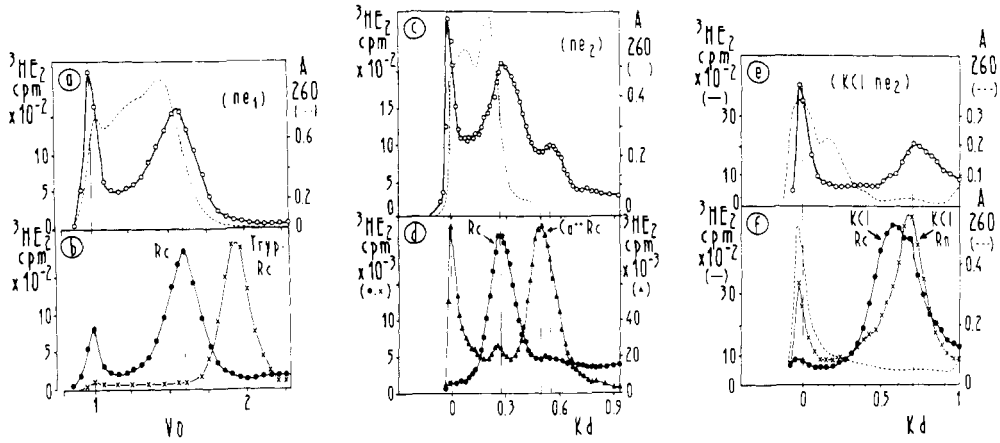

FIGURE 3: Gel filtration. The micrococcal nuclear extracts (NE_1 or NE_2, O) and the cytosol extracts containing the native (Rc, ●) or the partially proteolyzed cytosol receptors (Ca^{2+}Rc, ▲ TrypRc,*) were prepared as described under Materials and Methods and in Figure 1. The absorbance at 260 nm (- - -) and the radioactivity (—) were measured in aliquots of each fraction. (a, b) Sepharose 6B chromatography (total volume 150 mL). The number of void volume (V_0) was determined as indicated under Materials and Methods. The nuclear extract NE_1 contained 70% of the nuclear E_2R, 73% of which migrated as 4–6S peaks. The elution of cytosol samples started 4 h after their preparation, whereas that of NE_1 started 24 h later. (c, d) Ultrogel ACA 34 chromatography (total volume 200 mL). The distribution coefficient (K_d) was calculated from the relationship: $K_d = (V_e - V_0)/(V_t - V_0)$, where V_e = elution volume of each fraction; V_0 = void volume evaluated by Dextran blue; and V_t = total volume accessible to the solvent measured by β-mercaptoethanol. The elution of NE_2 or crude cytosol began 4 h after their preparation, whereas that of Ca^{2+}Rc-containing extract started 48 h after its preparation. (e, f) Ultrogel ACA 34 chromatography with buffer containing 0.5 M KCl. The micrococal nuclear extract (NE_2) (e), the cytosol (f), and the KCl nuclear extract (f) were analyzed as in c and d.

icance was not clear. It could possibly correspond to the $[^3H]E_2R$ bound to some chromatin material such as mononucleosome, subnucleosome, or nuclear proteins, but the occurrence of an artifactual aggregation of the receptor was not excluded. Actually, this peak was more prevalent than the 4–6S peak when chromatin was digested by lower concentrations of micrococcal nuclease (results not shown).

We thus concluded that an extended chromatin hydrolysis by micrococcal nuclease liberated the majority of the $[^3H]E_2$ Rn complexes which migrated in this extract as 4–6S peaks.

DNA Binding Ability of the Nuclear Receptor. We have previously shown that after partial proteolysis the cytosol receptor loses its DNA binding ability (André and Rochefort, 1973). The DNA binding ability of the nuclear receptor extracted by micrococcal nuclease was also evaluated in order to determine whether it displayed the properties of the native or of a partially proteolyzed receptor. The migration of the 4–6S nuclear receptor liberated by micrococcal nuclease was therefore investigated after incubation with soluble DNA either heavy (>15 S) or sonicated (~6 S) (Figure 2).

With heavy DNA, the 6S and 4S peaks disappeared, while the majority of the radioactivity was pelleted (Figure 2a). Moreover, 65 μg of heavy exogenous DNA displaced totally the $[^3H]E_2$ migrating with chromatin in extracts containing 250 μg of DNA, while they did not displace the nucleosome pattern. This result strongly suggested a better affinity of the RnE_2 complex for naked DNA than for slightly digested chromatin.

An aggregation of the receptor in the presence of exogenous DNA was unlikely, since the $[^3H]E_2Rn$ was displaced but not pelleted by the sonicated (6S) DNA, as shown in Figure 2b. Conversely, the 4S peak and the receptor bound to nucleosomes were only slightly displaced by this smaller DNA, contrary to what was observed with heavy DNA.

We conclude that the 4–6S nuclear receptors extracted by micrococcal nuclease digestion actually interacted with DNA but are unable to ascertain a difference of affinity for DNA of the 6S and 4S forms of the receptor.

During the same experiments, we determined that the cytosol receptor was actually able to interact with DNA (Figure 2c). Conversely, the 4S Ca^{2+}Rc (Figure 2d) was unable to bind DNA, since its pattern was not modified after addition of DNA. However, the different DNA-binding abilities observed could be due to other components in the extract rather than to the receptor itself. For instance, an "activating" factor could have been lost during the preparation of the Ca^{2+}Rc, or, conversely, the purification procedure could have enriched the Ca^{2+}Rc containing extracts in "inhibiting" factors. To check these hypotheses, we performed reconstitution experiments. On the one hand, the addition of nuclear extracts or cytosol to the Ca^{2+}Rc did not restore any DNA-binding ability of the Ca^{2+}Rc, thus confirming that this property was irreversibly lost during proteolysis (André and Rochefort, 1973). Conversely, the interaction of the 6S RnE_2 complex with heavy DNA was not prevented by adding the partially purified Ca^{2+}Rc which contained nonradioactive E_2 to occupy the cytosol receptor sites.

These results indicated that the DNA-binding ability of the RnE_2 complex solubilized by nuclease digestion and that of the $Ca^{2+}RcE_2$ were clearly different. They therefore strongly suggested that the RnE_2 was not derived from the partially proteolyzed Rc.

Gel-Filtration Analysis. The two nuclear extracts obtained after micrococcal treatment of nuclei isolated from labeled uteri were then analyzed by gel filtration in order to specify the Stokes radius of the RnE_2 complex under low ionic strength. With the NE_1 (Figure 3a) and NE_2 (Figure 3c) extracts, the majority of the radioactivity entered into the gel and did not comigrate with the chromatin material. This result suggested that the nuclear receptor which entered into the gel was not bound to chromatin. The proportion of the "free" RnE_2 measured by gel filtration was in agreement with the values obtained by ultracentrifugation analysis. Results were similar when using Sepharose 6B (Figure 3a) or Ultrogel ACA 34 chromatography according to Soulignac et al. (1977), and the same Stokes radius (5.2 nm) was found for the Rn localized in NE_1 and in NE_2. However, the free RnE_2 contained in NE_2 appeared more heterogeneous than that contained in NE_1, as also shown by ultracentrifugation analysis during the same experiment (Figure 1c).

We compared the elution pattern of the RnE_2 with those of the native and partially purified receptors (Figure 3b–d). In

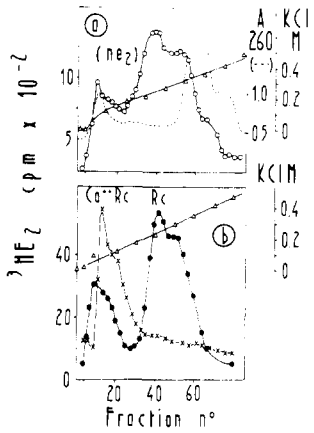

FIGURE 4: DEAE-cellulose chromatography. The micrococcal nuclear extracts NE_2 (a) and the cytosol (b) containing the native 8S Rc (●) or the 4S Ca^{2+}Rc (x) were prepared and analyzed on a DEAE-cellulose column as described under Materials and Methods. On each fraction, the KCl molarity (▲), the absorption at 280 nm (- - -), and the [^{3}H]E_2 content (—) were measured as described under Materials and Methods.

NE_1, the RnE_2 was eluted as the native Rc but clearly before the Rc partially proteolyzed by trypsin. In NE_2 (Figure 3c), the major peak entering into the gel was eluted just like the native Rc, whereas the minor peak migrated as the proteolyzed receptor, thus suggesting that it could be the result of an artifactual proteolysis of the receptor. The Stokes radius of the major peak of the nuclear R (5.2 nm) was not significantly different than that of the 8S R (5.3 nm) but was much higher than those of the Ca^{2+}Rc (2.9 nm) or the trypsin-Rc (3.0 nm). In order to rule out the possibility that the high Stokes radius observed for the nuclear receptor was due to interactions of a smaller nuclear receptor similar to the Ca^{2+}Rc with other molecules contained in the nuclear extract, we mixed unlabeled nuclear extracts with the 4S Ca^{2+} preparation. Under these conditions, the majority of the nonagregated 4S Ca^{2+}R was eluted at the same position with or without nuclear extracts (not shown). When the "micrococcal nuclear receptor" was treated by KCl and analyzed by Ultrogel chromatography in a KCl buffer (Figure 3e), the smaller Stokes radius obtained (2.8 nm) indicated that the nuclear receptor, like the 8S cytosol receptor and contrary to the partially proteolyzed receptor, had been dissociated from other components or into receptor subunits. In both cases, this dissociation was reversed by removal of the salt after Sephadex G-25 filtration. In a KCl medium, the Stokes radius of the dissociated "micrococcal Rn" was identical to that of the KCl Rn and of the 4S Ca^{2+}Rc (not shown) but smaller than that of the KCl-dissociated Rc (Figure 3e,f). The same dissociation process of the "micrococcal Rn" was also observed by sucrose gradient ultracentrifugation, where the 4–6S RnE_2 yielded a 3.8S peak in a KCl medium (not shown). The approximate anhydrous molecular weight of the "micrococcal Rn" could be evaluated from the Stokes radius and the sedimentation constant according to Siegel and Monty (1966):

$$\text{mol wt} = \left(\frac{6nN}{1 - vp}\right)sR_S = 4205sR_S$$

where n (solvent viscosity) is 0.01 P, p (density) is 1.00 g cm^{-3}, v (partial specific volume) is assumed to be 0.73 cm^3 g^{-1}, N is Avogadro's number, R_S is the Stokes radius in nanometers, and s the sedimentation constant in Svedberg units (10^{-13} s^{-1}). The apparent molecular weight of the "micrococcal" Rn was found to be slightly smaller than that of the 8S Rc but much higher than that of the partially proteolyzed receptor(s) (Table II). In a high salt medium, the molecular weight of the "micrococcal Rn" (45 000) was much smaller than that measured without salt (90 000 and 130 000), thus indicating a similar dissociation process to that observed in the cytosol. It also appeared to be slightly smaller than the usual cytosol or nuclear receptor treated and extracted by KCl. The molecular weights indicated in Table II are obviously relative and not absolute values, since they vary with the separation procedure and the degree of purity of the receptor (Jensen and De Sombre, 1972). The interest of these values is mainly a comparative one.

TABLE II: Comparison of the Nuclease-Extracted Nuclear Receptor with the Different Molecular Forms of the Cytosol Receptor.[a]

	micrococcal Rn	native Rc	Ca^{2+}Rc
sedimentat. const	6–4 S	8–6 S	~4.5 S
Stokes rad (nm)	5.2	5.3	2.9
calcd mol wt	130 000–90 000	170 000–130 000	55 000
DEAE-cellulose elut molarity by KCl	0.23 0.30	0.23 0.30	0.10
DNA-bind. ability	+	+	–
dissoc by KCl	+	+	–

[a] The results obtained with nonpurified R from lamb endometrium are represented. Similar molecular weights have been found for Rc by Soulignac et al. (1977) and for Ca^{2+}Rc by Puca et al. (1977). Similar elution molarity from the DEAE-cellulose has been reported by Puca et al. (1977).

DEAE-cellulose Chromatography. In a further comparison of the nuclear receptor and of the different forms of the cytosol receptor, each preparation was loaded onto a DEAE-cellulose column and eluted with a linear KCl gradient (Figure 4). A common striking point was that the radioactivity was never eluted as a single peak, suggesting a heterogeneity of the receptor. Most often, the saturable nuclear E_2 binding proteins present in NE_2 (Figure 4a) or NE_1 (not shown) were eluted as two broad peaks with 0.23 and 0.30 M KCl concentrations. The elution pattern was very similar to that obtained with the 8S Rc. The proportion of the two peaks varied according to the experiments both for the 8S cytosol and for the two micrococcal nuclear extracts, the major peak being eluted either at 0.23 or at 0.30 M KCl. In any case, the elution pattern was very different from that of the 4S Ca^{2+}Rc which was eluted at 0.10 M KCl (Figure 4, Table II). A similar difference between the 8S Rc and the Ca^{2+}Rc had already been reported in calf uterus (Puca et al., 1977).

We concluded that the ion-exchange chromatography gave additional evidence, indicating that the micrococcal nuclease extracted Rn was similar, if not identical, to the 8S Rc and clearly different from the Ca^{2+}Rc.

Discussion

The digestion of endometrial nuclei containing the E_2–nuclear receptor complex with micrococcal nuclease allowed us to extract the estradiol receptor under mild and low salt conditions. Up to 80% of the total DNA and of the saturable nuclear R could be solubilized by this procedure.

Two kinds of information were obtained, dealing firstly with the localization of the RnE_2 complex on chromatin and, secondly, with the relationship between the "micrococcal nuclear" receptor and the different forms of the cytosol receptor.

The in vivo localization of the RnE_2 complex on chromatin appears difficult to determine from in vitro biochemical studies, since a redistribution of the complex during the nuclear preparation and extraction is difficult to exclude. However, it is interesting to point out that after extended digestion of chromatin the majority of the E_2R was liberated from the

nucleosomes, while only 10% of DNA had been hydrolyzed into PCA-soluble material (see Results). Therefore, under these conditions, there was no parallelism between the migration of $[^3H]E_2$ and that of DNA involved in nucleosomes structures. Furthermore, the hydrolysis by nuclease of 10% DNA was sufficient to liberate up to 45% of the total RnE_2 complex from chromatin. Conversely, neither the lysis nor the sonication of the undigested nuclei was able to liberate more than 5% of the E_2R complex, suggesting that the nuclear receptor was not only trapped in the nuclear matrix, but actually interacted in vitro with nuclear material.

The nuclear receptor appeared to be weakly bound to nucleosomes, since it was totally displaced by the addition of soluble DNA (Figure 2). These results suggested that the RnE_2 complex was probably not interacting with the histone-DNA complex constituting the nucleosomes beads but could be mainly bound to DNA accessible to micrococcal nuclease and/or to non-histone-protein-DNA complex. Further experiments are obviously needed to determine the nature of the biological nuclear acceptor for the E_2R, since an in vitro redistribution of the RE_2 complex cannot be excluded.

When working under extensive chromatin digestion which produced more nucleosome monomers than polymers, the majority of the nuclear receptors which were liberated from the bulk chromatin could be directly characterized in a low salt medium, without extensive dialysis or gel filtration which are known to favor receptor agregation and proteolysis. Other attempts to characterize the nuclear R in a low salt medium had been previously performed. The removal of the salt after KCl extraction of the nuclei produced aggregates of the receptor complexes, and the 8-10S peak could be interpreted as the result of interaction with polyanions, as well as reassociation of subunits of the native receptor. The DNase I digestion of the 8-10S peak liberated a symmetrical 4S peak which could be the result of a partial proteolysis of R (Rochefort, 1970). The direct treatment of nuclei by DNase I and micrococcal nuclease has been shown to extract, respectively, 70 and 20% of the nuclear receptor from mouse uterus (Shyamala Harris, 1971). However, the RnE_2 contained in micrococcal nuclear extract was not analyzed in these studies, and the RnE_2 extracted by DNase I was found to be mostly aggregated. In order to obtain an 8S peak, the subsequent addition of polyanion was required. The biological meaning of these results is uncertain, since the *s* value of Rn was shown to vary according to the concentration of polyanions (Chamnes and Mc Guire, 1972). Recently, the digestion of nuclei with DNase II was used by Hemminki and Vaukkonen (1976, 1977). These investigators found that some nuclear estradiol receptor could be extracted, but the amount of the recovered receptor was too low to be analyzed further.

Extensive chromatin digestion at 2 °C gave us a better preservation of the E_2Rn and allowed us to determine some of its physicochemical parameters.

The homogeneity of this Rn is still uncertain, since after nuclear lysis at least two binding peaks (4S and 6S) were distinguished by sucrose gradient ultracentrifugation, gel-filtration analysis, and DEAE-cellulose chromatography. The significance of these two entities, either two different receptor subunits, A and B, as proposed by Schrader and O'Malley (1972) for the progesterone receptor, or a transformation of native receptor molecules into secondary receptor molecules formed either in vivo or artifactually in vitro according to an activation or inactivation process, was not determined here. It was, however, interesting to find constantly that the 6S entity was the most important in the first nuclear extract (NE_1), while the 4S peak was predominant in the second nuclear extract (NE_2), suggesting the occurrence of an artifactual splitting of Rn by nuclear protease. In addition, since only a part (up to 45%) of the total nuclear receptor was totally liberated from chromatin material, we cannot exclude the possibility that the nuclear receptor which was still binding to chromatin is different from that which has been analyzed.

In any case, the "micrococcal nuclear R" appeared clearly different from the partially proteolyzed cytosol R obtained either by trypsin (Erdos and Fries, 1974) or by Ca^{2+} (Puca et al., 1977) as far as their physicochemical properties are concerned (Table II). We have determined that the larger apparent molecular weight of the micrococcal Rn was not due to artifactual agregation of a proteolyzed R with other nuclear molecules. In addition, a functional difference was also noticed, since both forms of the micrococcal nuclear R were able to bind DNA, while this property is known to be irreversibly lost for the partially proteolyzed R (André and Rochefort, 1973). Therefore, these results support the hypothesis previously proposed (André and Rochefort, 1973; Vallet Strouvé et al., 1976), according to which the proteolyzed receptors were not able to be transferred to the nucleus. The proteolysis of R could be purely artifactual or might explain the biological inactivation of the receptor and its release from the nucleus.

Conversely, the "micrococcal Rn" was similar to the "native" 8S R, since it was also reversibly dissociated by KCl and displayed the same Stokes radius and DNA-binding ability (Table II) as the "native" molecule. However, its sedimentation constant is smaller than the 8S R. We do not know whether this difference is due to a different molecular weight or to a different conformation of the two R or whether it is secondary to interactions of R with different polyanions or proteins (Stancel et al., 1973). It is therefore proposed that the native cytosol R (6-8S) is translocated to the nuclei without a large modification of molecular weight. However, the hypothesis that the cytosol R is dimerized or associated with an X protein during the translocation (Notides and Nielsen, 1974) cannot be excluded, since it has been based on studies performed in high salt medium and on different animals. The analytical comparison in a KCl medium of the cytosol and nuclear R produces contradictory results depending on the authors and on the mode of analysis. Using a different mode of preparation of RnE_2, our conclusion agrees with that of Erdos and Fries (1974), who have shown that the molecular weights of the nuclear R and of the cytosol R were similar but much higher than that of the partially proteolyzed Rc. The major discrepancies found in the results of other laboratories could be due to minor but critical differences during the preparation and the analysis of the nuclear R concerning the temperature, the salt concentration, the degree of purification, and the principle of separation. Definitive conclusions will be obtained when the native R protein is purified to homogeneity from both the cytosol and nuclear compartments. However, the comparison of a nuclear R, prepared during this work, under mild, low salt conditions, with different molecular forms of the cytosol R, leads to the conclusion that the most likely form of the R involved in the nuclear translocation is the reversibly dissociable $8S \rightleftharpoons 4\text{-}5S$ R and favors the simplest hypothesis according to which no large modification of molecular weight is needed for the nuclear translocation step of R. Partial proteolysis of the R, such as that observed with trypsin or with the Ca^{2+}-transforming factor, cannot therefore be involved in the nuclear translocation step of the E_2 receptor.

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