Journal of Steroid Biochemistry, 1972, Vol. 3, pp. 445–458. Pergamon Press. Printed in Great Britain

ESTROPHILE TO NUCLEOPHILE IN TWO EASY STEPS

ELWOOD V. JENSEN, SURESH MOHLA, THOMAS GORELL, SEIEI TANAKA and EUGENE R. DESOMBRE

Ben May Laboratory for Cancer Research, The University of Chicago, Chicago, Illinois 60637, U.S.A.

SUMMARY

A variety of self-consistent experimental evidence supports the concept that estrogenic hormones interact with their target tissues by a multi-stage mechanism in which the hormone first associates with the 4S binding unit of an 8S extranuclear receptor protein, activating it to undergo conversion to a 5S form. This temperature-dependent transformation takes place when the receptor is bound to estradiol, but not when it is uncomplexed or bound to estrone. The transformed hormone-receptor complex migrates to the nucleus where it associates with an acceptor site in the chromatin. Analytical amounts of the 5S nuclear complex and a calcium-stabilized 4S cytosol complex have been isolated from calf uterus in apparently pure state, opening the way for the large scale purification of these different forms of the receptor protein.

The estrogen-receptor interaction sequence appears to be involved in the acceleration of biosynthetic reactions in hormone-dependent tissues. Exposure of isolated uterine nuclei either to transformed complex or to native complex under conditions where transformation can take place causes a significant increase in their ability to synthesize RNA. Similar treatment does not enhance the already high RNA synthetic capacity of liver or kidney nuclei. It is suggested that an important function of the hormone is to promote the conversion of the receptor protein to an active form which can enter the nucleus and alleviate a defficiency in RNA synthesis, characteristic of estrogen-responsive tissues.

ESTROGEN-RECEPTOR INTERACTION IN UTERINE TISSUE

EXTENSIVE studies over the past decade[1] have established that the characteristic affinity of estrogen-responsive tissues for estradiol is due to their content of specific hormone-binding macromolecules, commonly called "estrogen receptors" or "estrophiles". After exposure of a target tissue, such as uterus, to tritiated estradiol *in vivo* or at physiological temperature *in vitro*, most of the incorporated hormone is localized in the nucleus, from which it can be extracted by 0·3–0·4M potassium chloride as an estradiol-protein complex sedimenting in salt-containing sucrose gradients with a coefficient of about 5S. A smaller but significant portion (20–30 per cent) of the radioactive hormone appears to be extranuclear on autoradiography and is found in the cytosol fraction of a tissue homogenate, sedimenting as an 8S steroid-protein complex in sucrose gradients of low ionic strength and as a 4S complex in salt-containing gradients (Fig. 1).

The relation of these various estradiol-receptor complexes to each other is illustrated in Fig. 2. On entering the uterine cell, the hormone binds to an extranuclear receptor protein, which is present in amounts considerably greater than are utilized by a physiological dose of the hormone.* The actual form of this

*Immature rat uterus contains approximately 100 femtomoles of cytosol receptor per mg wet weight corresponding to about 100,000 receptor molecules per cell[2]. After a 0·1 μg dose of estradiol, the maximum level of incorporated hormone is about 20 femtomoles per mg[3]. After excision, much of the receptor capacity of uterine tissue is rapidly lost, so that, as usually prepared, homogenates of rat or calf uterus generally contain, respectively, about 50 and 30 femtomoles of receptor capacity per mg of tissue.

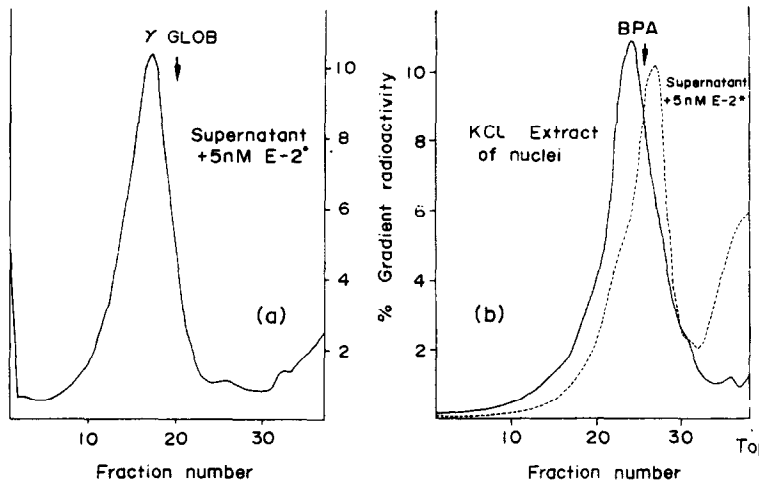

Fig. 1. Sedimentation pattern of radioactive estradiol-receptor complexes obtained from immature rat uteri. (a) Cytosol fraction of homogenate (in 10 mM Tris-1·5 mM EDTA, pH 7·5) of uteri excised from untreated animals, made 5 nM in estradiol-$_3$H (E-2*, 57 Ci/mmol) and centrifuged in a 10–30 per cent sucrose gradient at 308,000 *g* for 12 h at 1°C. (b) Similarly prepared cytosol plus 5 nM added E-2* (broken line) and 0·4 M KCl extract of nuclear sediment (solid line) from uteri excised from immature rats 1 h after subcutaneous injection of 100 ng (20·8 μCi) E-2* centrifuged in a 5-20 per cent sucrose gradient containing 400 mM potassium chloride at 284,500 *g* for 12 h at 2°C. Total CPM on gradient: (a) 30,240; (b) cytosol 35,380, nuclear extract 6035. γ-GLOB and BPA indicate sedimentation positions of γ-globulin (7S) and bovine plasma albumin (4·6S) markers. Part (b) reproduced from Jensen *et al.* [10].

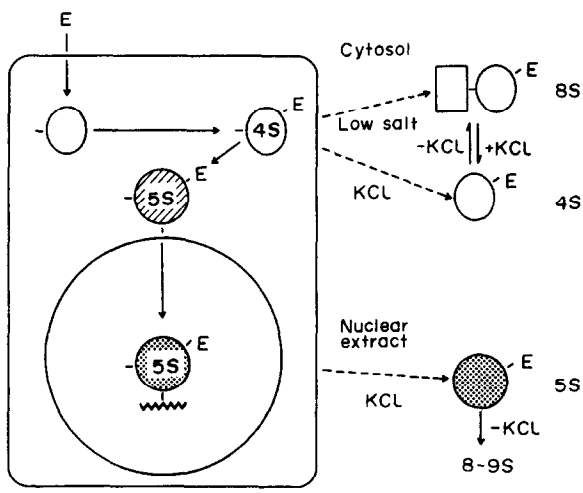

Fig. 2. Schematic representation of interaction pathway of estradiol (E) in uterine cells (see text).

receptor within the cell is not known; it appears to consist of a 4S binding unit, which under conditions of low ionic strength associates either with other binding units, with some nonbinding moiety or with both to give an 8S complex. Complexing with estradiol renders the 4S binding unit of the receptor susceptible to a

temperature-dependent conversion to a 5S form. This transformed complex migrates to the nucleus where it becomes attached to an acceptor site, apparently in the chromatin, from which it can be extracted by salt solutions.

The foregoing scheme for estrogen-receptor interaction is based on a variety of self-consistent experimental evidence. That there is some interrelation between cytosol and nuclear binding was first suggested by observations that *in vivo* both are reduced to the same degree by varying amounts of the specific binding inhibitor, nafoxidine[4], by considerations that the cytosol complex, which unlike the nuclear one is formed by direct exposure to estradiol, might serve as the non-saturable "uptake receptor" for saturable retention by the nucleus[5] and by findings that more radioactivity is taken up by uterine nuclei when they are incubated with tritiated estradiol in the presence of uterine cytosol than in its absence[6]. The mechanism outlined in Fig. 2 rests on the following observations: the absolute dependence on the presence of the cytosol receptor for 5S complex formation in isolated uterine nuclei[2, 7], the temperature-induced transfer of extra-nuclear to nuclear estradiol in uterine segments originally exposed to the hormone at 2°C *in vitro*[2, 8, 9], the temporary depletion of the uterine cytosol receptor which follows the administration of a physiological dose of estradiol *in vivo*[2, 10, 11], the estradiol-induced, temperature-dependent transformation of the binding unit of the cytosol receptor from a 4S to a 5S form[1. 12], and the striking difference between transformed and native cytosol complex in their affinities for uterine nuclei as described below.

The estradiol-induced depletion of cytosol receptor in the immature rat uterus is illustrated in Fig. 3. As indicated by three experiments, a minimum level is reached 4 h after hormone administration, after which the receptor content is gradually restored, apparently by resynthesis, inasmuch as the replenishment is prevented by the administration of cycloheximide. It is interesting that the total amount of 8S binding capacity which has disappeared at 4 h is four to five times greater than the amount of estradiol present in the nucleus at that time. If, as postulated, depletion of the cytosol receptor results from its transfer along with estradiol to the nucleus, this observation indicates that nuclear turnover of estradiol must be relatively rapid. It also suggests the possibility that on leaving the nucleus estradiol may encounter more receptor protein and repeat the process, so that each estradiol molecule may effect the transfer of several molecules of receptor protein to the nucleus.

When estradiol and uterine cytosol (but not estradiol alone) are incubated at 25–37°C with either crude nuclear sediment (Fig. 4) or sucrose-purified uterine nuclei (Fig. 5), subsequent extraction of the nuclei gives a 5S estradiol-receptor complex which is indistinguishable from that obtained after hormone administration *in vivo* or incubation of the whole tissue with estradiol *in vitro*. In contrast to estradiol, incubation of uterine nuclei with estrone and uterine cytosol does not give rise to extractable 5S complex (Fig. 4), even though estrone binds to the cytosol receptor to form an 8S complex or a 4S sub-unit depending on the ionic strength of the medium. This result is in accord with our previous finding that, after incubation of whole uterine tissue with estrone, the cytosol fraction contains 8S complex, but no 5S complex can be extracted from the nucleus (Fig. 6). It is evident that participation of a steroid in the sequential pathway of Fig. 2 requires more than its being able to bind to the extranuclear receptor protein to form the 8S complex.

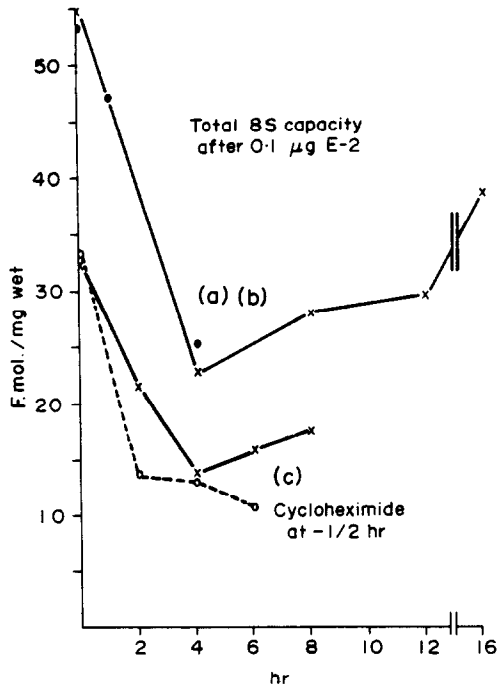

Fig. 3. Total estradiol-binding capacity of cytosol fraction of immature rat uteri excised at different times after subcutaneous injection of 100 ng estradiol-^{3}H in saline. Uteri (8–10 rats per group) were homogenized in the cold, and the supernatant fraction prepared by centrifugation at 300,000 *g* for 30 min at 2°, using four volumes of Tris-EDTA for experiment a (solid circles) and nine volumes for experiments b (crosses) and c (crosses and open circles). Cycloheximide-treated animals in c received 0·2 mg I.P. in 0·2 ml saline 30 min prior to the estradiol. After addition of excess E-2* (10-20 nM), a 200 μl aliquot portion of each cytosol was layered on a 5–20 per cent sucrose gradient containing Tris-EDTA, pH 7·4, and centrifuged for 6·5 h at 280,000 *g* or 8·5 h at 204,000 *g*. The total radioactivity in the 8S peak, plus small amount of aggregated material, was measured and expressed as femtomoles bound estradiol per milligram original uterine tissue. Concentration of estradiol in nuclear fraction at 4 h (in fmol/mg original tissue): a, 6·0; b, 6·2; c, 4·7. Reproduced from Jensen *et al.*[10].

The basis of this additional requirement is indicated by studies[1, 12] which demonstrate that conversion of the binding unit of the cytosol receptor from a 4S to a 5S form can take place in the absence of nuclei and appears to be a prerequisite for binding of the hormone-receptor complex in the uterine nucleus. When a mixture of cytosol and estradiol is incubated at temperatures of 25–37°C there is a progressive transformation of the receptor binding unit, as indicated by its sedimentation behavior in salt-contqining sucrose gradients (Fig. 7b). This conversion is not seen on incubating cytosol in the absence of hormone, nor does it take place when estrone is substituted for estradiol (Fig. 7a). Apparently the phenolic A ring of the estrone molecule can participate in binding to the receptor, but additional binding, involving the 17β-hydroxyl group, is required to promote transformation of the receptor. The characteristics of this transformation in the cytosol are entirely similar to those of the production of 5S complex in uterine nuclei incubated with estradiol and uterine cytosol: Both are temperature-dependent processes which are accelerated with increasing pH over the range

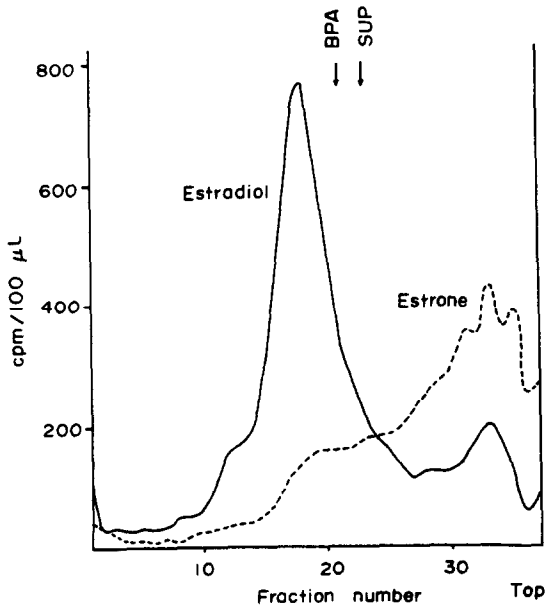

Fig. 4. Sedimentation pattern of potassium chloride extracts of nuclear sediment after incubation of a 10 per cent uterine homogenate in 10 mM Tris, pH 8·2, for 30 min at 25°C in the presence of 5 nM estradiol-^{3}H or estrone-^{3}H. Centrifugation was carried out in 5–20 per cent sucrose gradients containing 400 mM potassium chloride at 250,000 *g* for 16 h at 4°C. BPA and SUP indicate respective positions of bovine plasma albumin marker and 4S cytosol (supernatant) complex. (Experiment by Dr. Peter Brecher.)

6·5–8·5 and are inhibited but not prevented by the presence of EDTA; this inhibition is not relieved by Ca^{2+}, Mg^{2+} or Mn^{2+}, all of which themselves appear to exert a moderate inhibitory effect.

The transformed and untransformed estradiol-receptor complexes of uterine cytosol differ markedly in their affinities for uterine nuclei (Fig. 5). When incubated with sucrose-purified nuclei in the cold, an estradiol-cytosol mixture which has been previously warmed to 25°C to transform the complex gives a much greater incorporation of estradiol, extractable as 5S complex (Curve B), than does a mixture which has been kept at 2°C to prevent transformation (Curve D). When the incubation with nuclei is carried out at 25°C, the pretransformed receptor shows the greatest incorporation (Curve A), although the difference from native receptor (Curve C) is not so striking because, under these conditions, receptor transformation accompanies the nuclear uptake.

Whether the 5S complex extracted from the nucleus is identical with the transformed cytosol binding unit is not certain. On careful ultracentrifugation, the nuclear complex consistantly sediments slightly slower than the transformed cytosol complex, suggesting that a subtle further alteration may accompany the nuclear fixation of the transformed cytosol complex.

HORMONE-RECEPTOR COMPLEXES AND BIOLOGICAL ACTION

With the elucidation of the pattern of hormone-receptor interaction in uterine cells, the question arises as to the relation of this phenomenon to the hormonal induction and maintenance of tissue growth. That the binding of hormone to

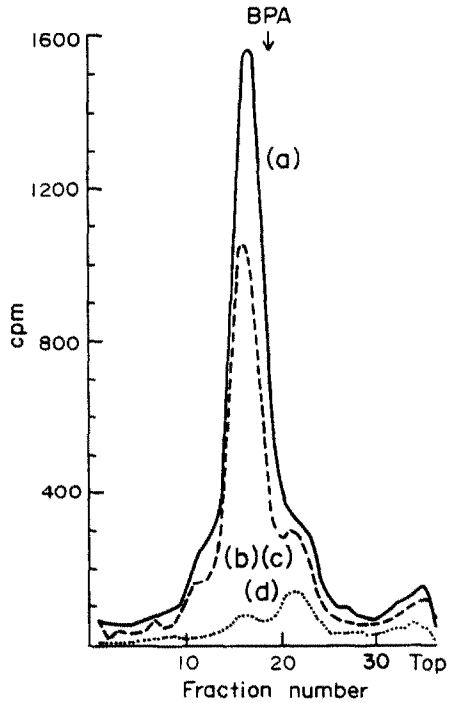

Fig. 5. Comparison of transformed and native cytosol complexes in their binding to sucrose-purified nuclei from calf endometrium. Endometrium cytosol, prepared in 0·32M sucrose, was made 5·6 nM in estradiol-^{3}H and incubated for 45 min at either 25°C (a and b) or 2°C (c and d). Equal portions of nuclei, purified by centrifugation in 2·2 M sucrose[35], were suspended in either the heated or the unheated estradiol-cytosol mixture and incubated for 60 min at either 25°C (a and c) or 2°C (b and d), after which the nuclei were separated, washed in sucrose and extracted with KCl. A 200 μl portion of each extract (containing in CPM: a, 11,200; b, 7,450; c, 7,430; d, 1,520) was centrifuged in a 5–20 per cent sucrose gradient containing 0·4M KCl at 317,000 *g* for 14 h at 2°C. The curves from experiments b and c were essentially superimposable.

receptor is intimately involved in the growth response was suggested very early by observations that the ability of various doses of nafoxidine to inhibit the uterine binding of estradiol *in vivo* closely parallels their inhibition of overall uterine growth[13].

The fact that estradiol moves to the nucleus together with receptor protein might suggest that the receptor system is simply a transport mechanism which delivers the hormone to its eventual site of action. On the other hand, it has been suggested[1] that the receptor protein itself, or the steroid–protein complex, may play a key role in some nuclear process and that the function of the hormone is to promote transformation of the protein to an active form which can enter the nucleus. Evidence for the latter possibility is provided by the striking effect of transformed but not untransformed estradiol-receptor complex in enhancing RNA synthesis in isolated uterine nuclei.

In 1969, Raynaud-Jammet and Baulieu[14] made the important observation that nuclei isolated from heifer endometrium, while not affected by estradiol or cytosol alone, show an increased ability to incorporate radioactive nucleotide into RNA after they have been incubated with a mixture of estradiol and uterine

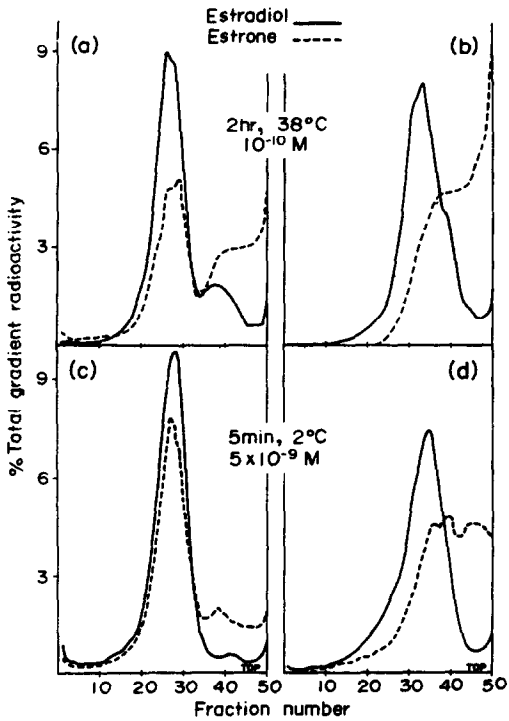

Fig. 6. Sedimentation patterns of cytosol (a, c) and nuclear extract (b, d) after *in vitro* exposure of immature rat uteri to estradiol (solid line) or estrone (broken line). In one experiment, uterine horns were slit lengthwise and stirred for 2 h at 38°C in Krebs–Ringer–Henseleit-glucose buffer, pH 7·3, containing either 0·1 nM estradiol-^{3}H or estrone-^{3}H; in a second experiment, incubation was carried out in 5 nM hormone for 5 min at 2°C. The groups of uteri were rinsed and homogenized in 4 volumes of cold 10 mM Tris buffer, pH 7·4, containing 1·5 nM EDTA; the homogenates were centrifuged 1 h at 216,000 *g* to separate cytosol and total particulate fractions and the latter extracted with 0·3M KCl at pH 7·5 (with freezing and thawing). Sedimentation of the cytosol complexes was carried out at 2°C for 7 h at 216,000 *g* in 5–20 per cent sucrose gradients; nuclear extracts were centrifuged similarly for 10 h in gradients also containing 0·3M KCl. The total radioactivity in each preparation (in DPM/100 μl) was: a: E-2, 4630; E-1, 2500; b: E-2, 8040; E-1, 2280; c: E-2, 42,180; E-1, 33,960; d: E-2, 8640; E-1, 7320. (Experiment by Dr. Tetsuro Suzuki.)

cytosol. Subsequent studies[15, 16] indicated that the RNA polymerase activity of heifer endometrium nuclei, or of the enzyme prepared from these nuclei, can be enhanced by adding a mixture of estradiol and certain uterine fractions directly to the polymerase system. We have been able to demonstrate[17, 18] that susceptibility of RNA synthesis to stimulation by the estradiol-receptor complex is a specific characteristic of nuclei from hormone-dependent tissues, such as uterus, and that only the transformed form of the complex is able to produce this activating effect.

After incubation at 25°C for 30 min with uterine cytosol in 2·2M sucrose, purified nuclei from immature rat uteri show a much lower ability to incorporate labeled nucleotide into RNA than do kidney or liver nuclei (Fig. 8). When 10 nM estradiol is also present during incubation, RNA synthesis in uterine nuclei is increased nearly 3 fold, whereas there is no enhancement of the already high

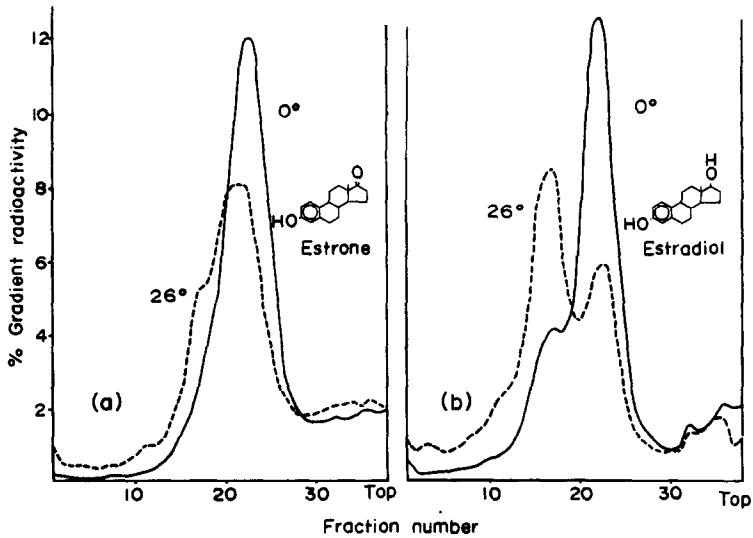

Fig. 7. Comparison of estrone and estradiol in promoting conversion of 4S to 5S complex. Uterine horns from 22-day old rats were homogenized in nine volumes of 10 mM Tris buffer, pH 7·5. Portions (0·9 ml) of the supernatant fraction were treated with 0·1 ml of buffer containing: (a) 20 nM estrone-^{3}H or (b) 20 nM estradiol-^{3}H to give a final hormone concentration of 2 nM. A portion of each mixture was incubated for 30 min at either 26 or 0°C, after which 200 μl aliquots were layered on 5–20 per cent sucrose gradients containing 400 mM KCl and centrifuged at 2°C for 15 h at 308,000 *g*. Total CPM in the gradients were 10,490 and 10,720 for estrone and 11,650 and 11,200 for estradiol. Reproduced from Jensen *et al.*[1].

synthetic capacity in kidney or liver nuclei after incubation with hormone, either in uterine cytosol or in their own cytosols. Thus, nuclei from the hormone-dependent tissue appear to possess a deficiency in RNA synthetic capacity which can be alleviated by treatment with the estradiol-receptor complex of uterine cytosol.

Stimulation of uterine nuclei requires hormone-induced conversion of the receptor protein binding unit from the 4S to the 5S form (Table 1). Nuclei from calf endometrium are activated by incubation with estradiol and endometrium cytosol at 25°C, where receptor transformation takes place readily, but not at 0°C, where it does not. However, if the estradiol-cytosol mixture is first warmed to 25°C to effect transformation of the receptor, the resulting 5S complex can stimulate nuclei on incubation at either 0 or 25°C. These results are in accord with the ability of transformed but not untransformed complex to bind to uterine nuclei, even in the cold (Fig. 5). Estrone, which is known to form the 4S complex but is unable to induce its conversion to the 5S form (Fig. 7), does not cause nuclear stimulation under conditions where estradiol is effective. The 5S estradiol-receptor complex, extracted from calf uterine nuclei previously incubated with estradiol and uterine cytosol, is also active in stimulating fresh nuclei.

The magnesium-dependent RNA polymerase activity of mammalian nuclei prepared in 2·2M sucrose can be separated into two fractions: bound enzyme, firmly associated with chromatin, and soluble enzyme, which is extracted from the nuclei by 0·32M sucrose[19]. It was found that both types of RNA polymerase activity are enhanced after incubation of endometrial nuclei with the

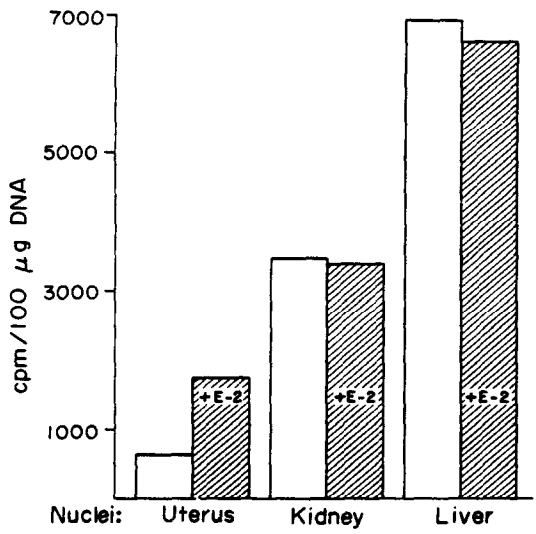

Fig. 8. RNA synthesis in nuclei isolated in 2·2M sucrose [35] from various rat tissues after incubation with rat uterine cytosol for 30 min at 25°C in 2·2M sucrose in presence and absence of 10 nM estradiol. After incubation, the nuclei were separated by centrifugation and resuspended for polymerase assay in 0·32M sucrose containing 3 mM $MgCl_2$ in 10 mM Tris, pH 7·4. This assay, essentially the procedure of Weiss [36], is based on the net incorporation of radioactivity from tritiated UTP into acid-insoluble residue during 10 min at 37° in a Mg^{2+}, ATP, CTP, GTP system, described in detail elsewhere [18]; blank determinations contained 0·12M EDTA. Results expressed on basis of DNA in nuclear suspension, determined colorimetrically [37].

Table 1. RNA synthesis in calf endometrium nuclei

Expt.	Nuclei incubated with:	Incubation Temp. (°C)	Subsequent UMP-H^3 into RNA (% of control)
1a	Cytosol + estradiol	25	220*
b	Cytosol + estradiol	0	95
c	Cytosol + estradiol (pretransformed at 25°)	25	240*
d	Cytosol + estradiol (pretransformed at 25°)	0	270*
2a	Cytosol + estradiol	25	170*
b	Cytosol + estrone	25	105
c	Sucrose + estradiol	25	90
3a	Cytosol + estradiol	25	195*
b	Nuclear extract	25	235*
c	Nuclear extract	0	175*

Incubations were carried out in 2·2M sucrose for 30 min (expts. 1 and 2) or 45 min (expt. 3). In expt. 3, 35 mM KCl was also present. Estradiol and estrone were 10 nM. In expts. 1c and 1d, receptor transformation was effected by incubating the estradiol–cytosol mixture at 25°C for 30 min before the nuclei were added. Control values were obtained with cytosol without steroid, except in expts. 3b and 3c where heat-inactivated (50°C, 15 min) nuclear extract was used for the control. Experiments involving transformed receptor are indicated by an asterisk.

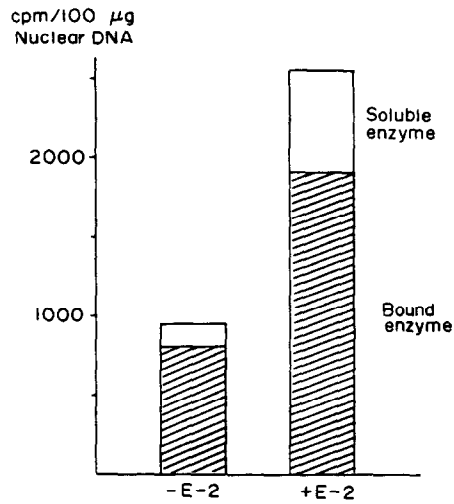

Fig. 9. RNA synthesis by soluble and bound RNA polymerase of calf endometrium nuclei after incubation of 2·2M sucrose homogenate for 35 min at 25°C in the presence and absence of 10 nM estradiol. After incubation, the nuclei were separated by centrifugation and suspended for 30 min at 2°C in 0·32M sucrose-1 mM MgCl in 20 mM Tris, pH 7·5, to extract the soluble enzyme[19]. After centrifugation, the nuclei with the bound enzyme were resuspended for assay in 0·32M sucrose-3 mM $MgCl_2$ as described for Fig. 8. The soluble enzyme in the extract was assayed similarly, using calf thymus DNA as template; blanks for the soluble enzyme contained no added DNA.

estradiol-cytosol mixture (Fig. 9). This still preliminary observation suggests that at least part of the ability of transformed estrogen-receptor complex to stimulate RNA synthesis in uterine nuclei involves an action other than an effect on chromatin template activity. This conclusion is in agreement with the findings of Barry and Gorski[20] that the increased rate of precursor incorporation into RNA in uterine nuclei isolated from estrogen-treated rats appears to result from the synthesis of longer RNA chains, rather than more chains which would be the case if new template sites were being made available by hormonal treatment.

The degree of enhancement (50–200 per cent) of RNA synthetic capacity of uterine nuclei when they are treated directly with the estradiol-receptor complex is comparable to that observed in uterine nuclei after giving estrogen *in vivo* [21–23] and is considerably greater than that which would correspond to new messenger for a single protein species. Thus, the relation of this effect to the early formation of the "induced protein" in rat uterus, as described by other investigators[24–29], is not clear. Still the tissue and hormone specificity associated with this stimulation of nuclear RNA polymerase, and its correlation with the transformed receptor complex, suggests that the phenomenon is of physiological significance and that it may provide a valuable system for evaluating the biological activity of purified receptor proteins when these become available.

PURIFICATION OF RECEPTOR PROTEINS

A clearer understanding of the biochemical role of the estrogen receptor protein, the chemical basis of the 4S to 5S transformation and the relation between the 8S protein and its 4S binding unit should be possible when the various forms of the receptor substance are available in a pure state in amounts sufficient

to permit determination of chemical composition and biochemical properties. We have now succeeded in isolating, in apparently pure form, microgram amounts of both a calcium-stabilized 4S estradiol-receptor complex of calf uterine cytosol and the 5S nuclear complex obtained by incubating calf uterine nuclei with estradiol in uterine cytosol.

Because of its instability and tendency toward aggregation, isolation of the purified cytosol receptor protein has proved difficult. Purification of the binding unit of the cytosol receptor was facilitated by the observation [30, 31] that addition of calcium ions to the salt-dissociated complex of uterine cytosol, prepared in the presence of EDTA, yields a "stabilized" 4S binding unit which does not revert to the 8S form when the salt is removed and which is highly resistant to aggregation. Although the 4S complex thus prepared no longer will undergo transformation to the 5S form, it provides the essential binding unit of the cytosol receptor in a form which can be purified by convential techniques of protein chemistry.

By ammonium sulfate precipitation, Sephadex G-200 filtration and DEAE-

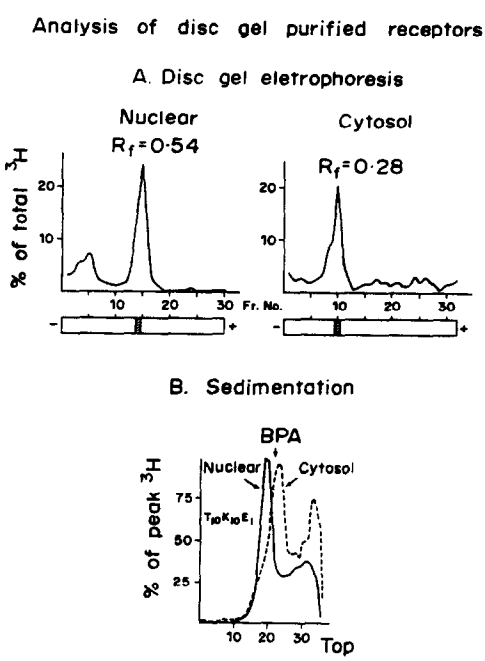

Fig. 10. Analysis of purified estradiol-receptor complexes. Preparations of calcium-stabilized cytosol receptor complex (4S) and nuclear complex (5S), each previously purified by ammonium sulfate precipitation, gel filtration on Sephadex G-200, and ion exchange chromatography on DEAE-cellulose were subjected to preparative disc gel electrophoresis on acrylamide gel, followed by slicing of the gels and elution of the tritium-containing area at 2°C with buffer (10 mM Tris, 30 mM KCl, 1 mM EDTA, pH 7·4). Portions of each eluate were then subjected to analytical disc gel electrophoresis in duplicate (A); one gel was sliced, dried and combusted to give the tritium distribution, as shown in the upper figures, and the other stained with amido black to give the protein distribution as shown directly below. A third portion (200 μl) of each eluate was layered on a linear, preformed 10–30 per cent sucrose gradient containing 10 mM KCl (B); after centrifugation for 16 h at 300,000 g (nuclear) or 308,000 g (cytosol), the sedimentation patterns were compared with that of a BPA marker.

cellulose chromatography, the calcium-stabilized 4S complex of calf uterine cytosol, prepared by high speed centrifugation, has been purified about 5000 fold and that from low-speed cytosol about 1000 fold, corresponding to respective purities of about five per cent and one per cent, if one estradiol is bound per 4S unit[31]. This partially purified 4S complex shows an apparent molecular weight (G-200 elution) of about 75,000 and an isoelectric point of 6·4, in contrast to respective values of 200,000 and 5·8 observed with the 8S cytosol complex. Subsequent studies by Puca[32], involving determination of Stokes radii, indicate molecular weights of 236,000 for the 8S complex and 61,000 for the stabilized 4S complex, as well as isoelectric points of 6·6–7·0 and 6·2, respectively. As illustrated in Fig. 10, the product from low speed cytosol has been further purified by repeated acrylamide gel electrophoresis to yield a 4S complex showing a single radioactive protein band by amidoblack staining[33].

Under most conditions, the 5S complex extracted from the nucleus undergoes aggregation to an 8S to 9S form when the salt is removed. Unlike the binding unit of the cytosol complex, the nuclear complex is not stabilized toward aggregation by treatment with calcium ions. After ammonium sulfate precipitation followed by gel filtration in the presence of salt, the nuclear complex loses its tendency to aggregate in low salt and can be further purified by ion-exchange chromatography and/or acrylamide gel electrophoresis at pH 8·8, where it moves considerably faster than the calcium-stabilized 4S complex of the cytosol. In this way the nuclear complex, prepared by incubating crude calf uterine nuclei with estradiol and cytosol, has been obtained as a single radioactive band on gel electrophoresis [34]. As illustrated in Fig. 10, this purified 5S nuclear complex shows distinct differences from the 4S cytosol complex, both in sedimentation and electrophoretic properties.

Although the foregoing experiments have yielded the purified 4S and 5S complexes in what probably are only microgram amounts, current attempts to scale up the preparative procedures should be capable of yielding these products in tangible quantity. It is hoped that after the 4S cytosol complex is available in amounts sufficient to produce specific antibodies such antibodies will cross-react with the 8S complex and thus may be utilized for the selective isolation of the intact cytosol receptor, a substance which has proved difficult to purify by conventional means.

ACKNOWLEDGEMENTS

Various aspects of these investigations were supported by grant 690-0109 from the Ford Foundation, grant P-422 from the American Cancer Society, U.S. Public Health Service grant CA-02897 from the National Cancer Institute and contract NIH-69-2108 from the National Institute of Child Health and Human Development. One of us (E. R. DeSombre) is supported by Research Career Development Award HD-46, 249 from the NICHD.

REFERENCES

1. A more detailed summary of the many studies on estrogen-receptor interaction with specific reference to the original publications is given in: E. V. Jensen, M. Numata, P. I. Brecher and E. R. DeSombre: In *The Biochemistry of Steroid Hormone Action*. Biochemical Society Symposium No. 32 (Edited by R. M. S. Smellie). Academic Press, London (1971) pp. 133–59.
2. E. V. Jensen, T. Suzuki, T. Kawashima, W. E. Stumpf, P. W. Jungblut and E. R. DeSombre: *Proc. Natn. Acad. Sci. U.S.A.* **59** (1968) 632.
3. E. V. Jensen and H. I. Jacobson: *Recent Progr. Hormone Res.* **18** (1962) 387.
4. E. V. Jensen, E. R. DeSombre and P. W. Jungblut: In *Hormonal Steroids* (Edited by L. Martini, F. Fraschini and M. Motta). Excerpta Med. Found., Amsterdam (1967) pp. 492–500.

5. E. V. Jensen, E. R. DeSombre, D. J. Hurst, T. Kawashima and P. W. Jungblut: *Arch. Anat. Microscop. Morphol. Exp. suppl.* **56** (1967) 547.
6. P. I. Brecher, R. Vigersky, H. S. Wotiz and H. H. Wotiz: *Steroids* **10** (1967) 635.
7. T. A. Musliner, G. J. Chader and C. A. Villee: *Biochemistry* **9** (1970) 4448.
8. J. Gorski, D. Toft, G. Shyamala, D. Smith and A. Notides: *Recent Progr. Hormone Res.* **24** (1968) 45.
9. G. Shyamala and J. Gorski: *J. biol. Chem.* **244** (1969) 1097.
10. E. V. Jensen, T. Suzuki, M. Numata, S. Smith and E. R. DeSombre: *Steroids* **13** (1969) 417.
11. M. Sarff and J. Gorski: *Biochemistry* **10** (1971) 2557.
12. P. I. Brecher, M. Numata, E. R. DeSombre and E. V. Jensen: *Fedn. Proc.* **29** (1970) 249.
13. E. V. Jensen: *Proc. Canadian Cancer Res. Conf.* **6** (1965) 143.
14. C. Raynaud-Jammet and E. E. Baulieu: *Compt. rend. (Paris)* **268D** (1969) 3211.
15. Y. Beziat, J. C. Guilleux and M. Mousseron-Canet: *Compt. rend. (Paris)* **270D** (1970) 1620.
16. M. Arnaud, Y. Beziat, J. C. Guilleux, A. Hough, D. Hough and M. Mousseron-Canet: *Biochim. biophys. Acta* **232** (1971) 117.
17. S. Mohla, E. R. DeSombre and E. V. Jensen: *Fedn. Proc.* **30** (1971) 1214.
18. S. Mohla, E. R. DeSombre and E. V. Jensen: *Biochem. biophys. Res. Commun.* **46** (1972) 661.
19. S. Liao, D. Sagher and S. Fang: *Nature* **220** (1968) 1336.
20. J. Barry and J. Gorski: *Biochemistry* **10** (1971) 2384.
21. T. H. Hamilton, C. C. Widnell and J. R. Tata: *J. biol. Chem.* **243** (1968) 408.
22. J. Gorski: *J. biol. Chem.* **239** (1964) 889.
23. J. A. Nicolette, M. A. Lemahieu and G. C. Mueller: *Biochim. biophys. Acta* **166** (1968) 403.
24. A. Notides and J. Gorski: *Proc. Natn. Acad. Sci. U.S.A.* **56** (1966) 230.
25. R. F. Mayol and S. A. Thayer: *Biochemistry* **9** (1970) 2484.
26. A. Barnea and J. Gorski: *Biochemistry* **9** (1970) 1899.
27. A. B. DeAngelo and J. Gorski: *Proc. Natn. Acad. Sci. U.S.A.* **66** (1970) 693.
28. B. S. Katzenellenbogen and J. Gorski: *Fedn. Proc.* **30** (1971) 1214.
29. C. R. Wira and E. E. Baulieu: *Compt. rend.* **273D** (1971) 218.
30. E. V. Jensen, M. Numata, S. Smith, T. Suzuki, P. I. Brecher and E. R. DeSombre: *Develop. Biol. suppl.* **3** (1969) 151.
31. E. R. DeSombre, G. A. Puca and E. V. Jensen: *Proc. Natn. Acad. Sci. U.S.A.* **64** (1969) 148.
32. G. A. Puca, E. Nola, V. Sica and F. Bresciani: In *Advances in the Biosciences* (Edited by G. Raspé). Pergamon-Vieweg, Braunschweig Vol. 7 (1971) pp. 97–113.
33. E. R. DeSombre, J. P. Chabaud, G. A. Puca and E. V. Jensen: *J. steroid Biochem.* **2** (1971) 95.
34. E. R. DeSombre, M. Ikeda, S. Tanaka, S. Smith and E. V. Jensen: Abstracts 53rd Meeting The Endocrine Soc., San Francisco (1971) p. 149.
35. J. Chauveau, Y. Moule and C. Rouiller: *Exp. Cell Res.* **11** (1956) 317.
36. S. B. Weiss: *Proc. Natn. Acad. Sci. U.S.A.* **46** (1960) 1020.
37. K. Burton: *Biochem. J.* **62** (1956) 315.

DISCUSSION

Rosner: Dr. Jensen, you mentioned that a given estradiol molecule migrates back and forth, forms more complex and comes back in, allowing more protein to get into the nucleus. How do you visualize that? Baulieu has recently shown that the half-life of dissociation of the estradiol-cytosol receptor, depending on which of his figures you looked at, was between 10 and 20 days at 4°C.

Jensen: That's in an *in vitro* system. We know nothing about how the receptor or the hormone get out of the nucleus *in vivo*. I only suggest that estradiol might be recycling as an explanation for the fact that one observes a larger disappearance of cytosol receptor than can be accounted for by the estradiol present in the nucleus.

Adlercreutz: Have you tried any of the common synthetic estrogens?

Jensen: Yes. The transformation of the 4S to 5S form can be induced by estradiol, 17-ethynylestradiol, stilbestrol, hexestrol, and estriol. It is not brought about by estrone, as I already mentioned, and it is also not effected by compounds like

mestranol or quinestrol, which are 3-methyl ethers. In fact, when you block the 3 position, these substances don't even form the 4S complex.

Munck: What's approximately the relative affinity of estrone compared to estradiol for the cytosol receptor?

Jensen: Maybe about 5 to 1. Estrone binds quite well, but definitely not as well as estradiol. Estradiol competes with estrone binding better than estrone with estradiol binding.

Wira: What's the earliest time that you were able to observe new synthesis of cytosol receptor? I noticed in one of your slides that you added cycloheximide, and found a block of cytosol receptor.

Jensen: Right. The content of receptor in the cytosol reaches a minimum at about 4 h in the immature animal. In the mature ovariectomized animal, you get the same effect, but it reaches a minimum at about 6 h, quite reproducibly. One sees the content coming back up again after 4 h; however, I think resynthesis is probably stimulated earlier than this. One has two effects during the first 4 h: the receptor is being used up and resynthesis begins. It's only when resynthesis outruns depletion that the level starts coming up. If you remember, the cycloheximide curve lay below the non-inhibited one even at the 1 h time point, indicating that there is already some resynthesis within 1 h which is being blocked by cycloheximide.

Wira: My reason for asking this question is related to the possible role of the estradiol-induced protein (IP) which Dr. Gorski found in *in vivo* studies and which we have more recently observed following *in vitro* stimulation with 10^{-9} M estradiol. Since we observe the synthesis of IP initially at 1 h, I was wondering if at least one component, since the IP may represent a family of proteins, might in fact be the beginning of newly synthesized cytosol receptor.

Jensen: I think this is a very good suggestion, because this idea has occurred to us, too. Looking at the properties of induced protein, we were intrigued by the possibility that maybe this could be the receptor substance. We have been thinking about how to test this experimentally.