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ACTIVATION OF TRANSCRIPTION-REGULATING PROTEINS BY STEROIDS*

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SUMMARY

The intracellular proteins, which bind steriod hormones with high affinity and specificity have been generally considered as instruments of hormone action. A reversal of assignments might seem a merely semantic exercise, but is indeed in better agreement with experimental evidence identifying 'receptors' as transcription-regulating proteins. The series of events in the presence of hormone are:

1. attachment of the steroid to the 'receptor' which undergoes a major conformational change when 'enveloping' the steroid,
2. dimerization to steroid-receptor: ɹoɪdəɔəɹ-pɹoɹəɪs
3. translocation of the dimer into the nucleus,
4. enhancement of transcription.

One product of the latter is 'receptor' mRNA, the translation of which initiates within 60 - 90 min after pulse-administration of steroid. In the absence of hormone, 'receptor translocation', degradation and biosynthesis continue to proceed but at a much slower rate. Although these results have been primarily obtained with the estradiol-'receptor' system, all other systems seem to follow the same pattern. The molecular mechanism by which enhancement of transcription is achieved is as yet unknown. Its specificity must be quite particular since several steroid-'receptor' systems occur simultaneously within the same cell.

INTRODUCTION

Ever since the discovery of 'receptors', our state of knowledge on the mechanism of action of steroid hormones has been rapidly progressing. It is quite understandable that, when speaking of the two reactants, more emphasis usually was placed on the importance of the hormone. A typical example for this practise is the still frequently used phrase "the transport of the steroid to (into) the nucleus by the cytoplasmic receptor". Isidore Edelman's statement at the Schering Workshop on Steroid Hormone Receptors [1], that it remained to be shown "who carries whom", was probably the first doubt to be shed on this ranking order. The statement drew little attention then, since both the hormone and the receptor were thought of as indispensable elements for the full course of action. Although receptor-independent effects of steroid hormones were and remain conceivable, no hormone-independent function of receptors was envisaged. This paper reports on data, which are not compatible with this view and therefore justify a reappraisal of the situation.

*Dedicated to Charles B. Huggins on the Occasion of his 75th birthday.

MATERIALS AND METHODS

The following radioactive steroids were used: [6.7-^{3}H]-estradiol, S.A. 42.6 Ci/mmol; [6.7-^{3}H] -R 5020 (17.21-dimethyl-19-nor-4.9-pregnadiene-3.20-dione. Roussel-UCLAF); 51.4 Ci/mmol. Other chemicals were of analytical grade.

Animals and experimental procedures: Sprague - Dawley rats were used throughout, operated on at least three weeks before the experiments: priming, when carried out, consisted of 4 × 1 μg estradiol/0.5 ml sesame oil sc, each second day, last injection 3 days before experiment. Intra-uterine injections were performed under ether anesthesia, 20 μl of the test solution being injected *via* the cervix using a Hamilton syringe with a constant vol. dispenser coupled to a blunted 22 gauge needle. For circadian rhythm experiments, rats were trained daily for 8 days prior to experiment.

German Landrace pigs were ovariectomized and the uteri modified at 3 - 4 months of age. Pigs were primed by the sc implantation of silastic tubing containing a crystalline suspension of estradiol in propylene glycol. Implants were removed 8 days prior to experiment. Intra-uterine injections (20 ml of test solution) were carried out on unanesthetized pigs, previously trained by a series of sham injections coupled with rewards of sweet beer.

Uterine extracts were prepared following dismembration (rats, 1 + 4 tissue: buffer) or ultra-turrax treatment (pigs, 1 + 1 tissue: buffer). Cytosols were

obtained by high-speed centrifugation of homogenates (SW 40 or SW 56, Beckman L2-65B). Microsomal fractions and extracts were prepared as described previously [2]. Nuclei were isolated by a procedure involving differential centrifugation and multiple sievings, and were stripped by exposure to 0.1% Triton x-100. Nuclear receptors were extracted in buffer containing 0.3 M KCl, 0.05 M DTT and 6×10^{-8}M labelled estradiol.

Cytosols, microsomal and nuclear extracts were analysed by density gradient centrifugation (5 - 20% or 10 - 30% sucrose gradients at various pH's collected by constant vol. sampling) and by agar electrophoresis [3]. Radioactivity was counted in Packard Tri-Carb 3320 with an efficiency for tritium of about 40%, in a xylene:dioxane based fluor [2].

RESULTS AND DISCUSSION

The activation of the estradiol-receptor by hormone-facilitated dimerization

In searching for the site of receptor biosynthesis, we extracted two proteins from the microsomal fraction of pig uteri, both binding estradiol with the same high affinity, but differing in electrophoretic mobility and sedimentation velocity [2]. The less polar protein, sedimenting at 3.5 *S*, was suspected to be an early product of receptor biosynthesis, already possessing the specific binding site and possibly representing the receptor core. The other protein was assumed to be identical with the 4 *S* cytosol receptor, since it displayed the same electrophoretic mobility and had an only slightly higher sedimentation coefficient of 4.5 *S*. Attempts to convert the electrophoretic mobility of the 'acidic' receptor to that of the 'basic' one by neuraminidase treatment had an unexpected result [4]. Instead of a change in the electrophoretic pattern, we observed a shift of the 3.5 *S* estradiol - receptor complex to the 4.5 *S* position on density gradient centrifugation. This occurred following incubation of the estradiol-containing extracts at 30° C, without the enzyme present. Kinetic analysis of the transition revealed a dimerization as the underlying mechanism. Stability studies indicated that histidyl and tyrosyl residues are essential for the hook-up. They further showed that the 4.5 *S* acidic microsomal receptor was not identical with the cytosol receptor, but was the dimer of an 'acidic' 3.5 *S* monomer. After lowering the pH of a heated extract from pH 7.0 to 6.5, the single 4.5 *S* peak disappeared, to be replaced by a peak sedimenting in the 3.5 *S* position. Renewed dimerization not only required readjustment of the pH, but also incubation at the same elevated temperature as that required for the original extract prepared at 0° C with low ionic strength buffer containing estradiol. Since the replacement of estradiol by estrone in the extractant gave rise to a 3.5 *S* peak only, composed of both 'basic' and 'acidic' receptors, a close structural apposition of 3.5 *S* acidic monomers in the cell, favouring the estradiol-mediated dimerization even

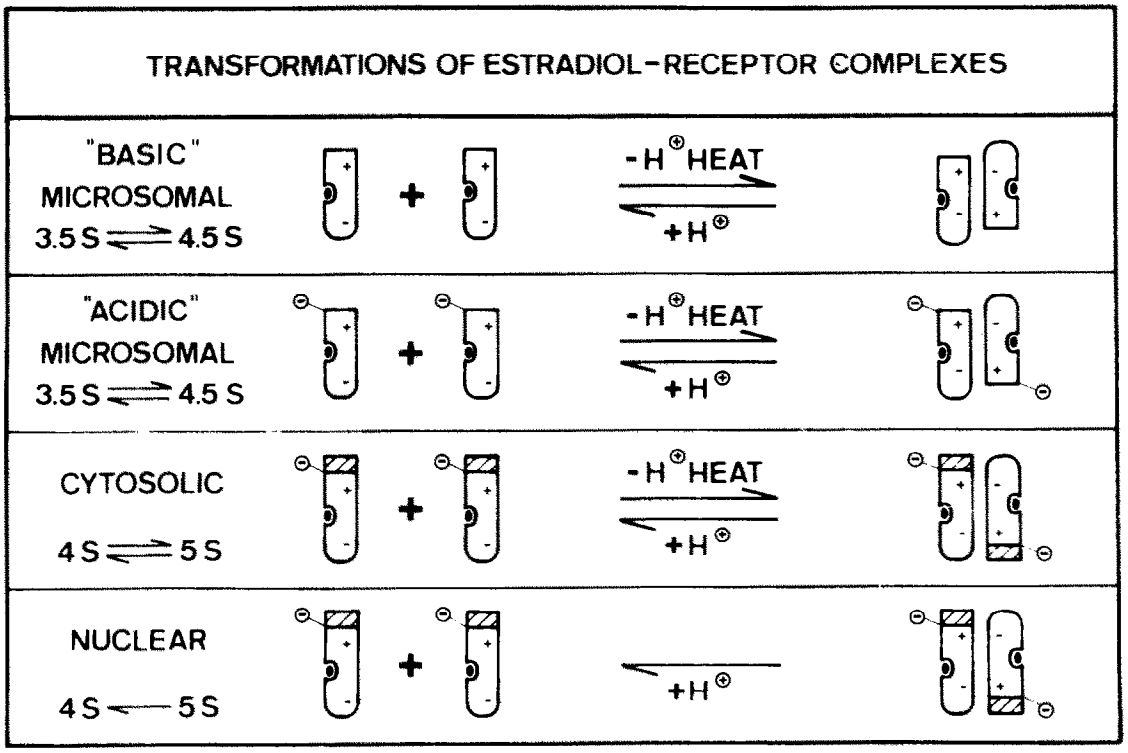


Fig. 1.

at a low temperature, is a more likely explanation for the occurence of the 4.5 *S* acidic dimer in cold extracts than the pre-existence of a dimer.

A similar phenomenon had previously been observed in cytosols of rat uteri by Brecher *et al.* [5, 6]. After addition of 0.4 M KCl and warming, the 4 *S* estradiol - receptor peak sedimented at the 5 *S* position known for the hormone receptor complex extracted from nuclei [5, 6]. This transition has been studied in detail by Notides and Nielsen [7] and in our laboratory [8]. It follows, as was found for the microsomal receptors, second order kinetics for dimerization, although these receptor monomers from the two cytoplasmic compartments are distinctly different apart from their steroid binding core.

In discussing their results, Notides and Nielsen, followed by Yamamoto and Alberts [9] were correct in not excluding the possibility that the dimerization might involve one receptor molecule and a second non-steroid binding entity of similar size and shape. Dealing with three different receptor monomers from two cytoplasmic compartments, we considered the presence of 'matching' non-binders in equimolar concentrations to be very unlikely and therefore

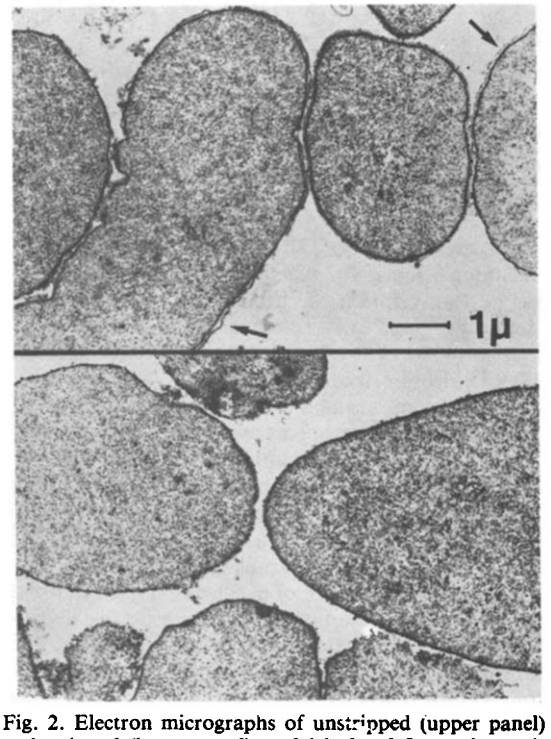


Fig. 2. Electron micrographs of unstripped (upper panel) and stripped (lower panel) nuclei isolated from pig uteri. Arrows indicate double membrane.

favoured the receptor-dimer concept. We can now add the receptors from a third compartment to this list (Fig. 1). Estradiol - receptor complexes extracted from nuclei, devoid of the rough ER-like outer layer of their envelope (Fig. 2), which can contain microsomal receptors, sediment at 5 *S*, this being reduced to 4 *S* after proton addition (Fig. 3).

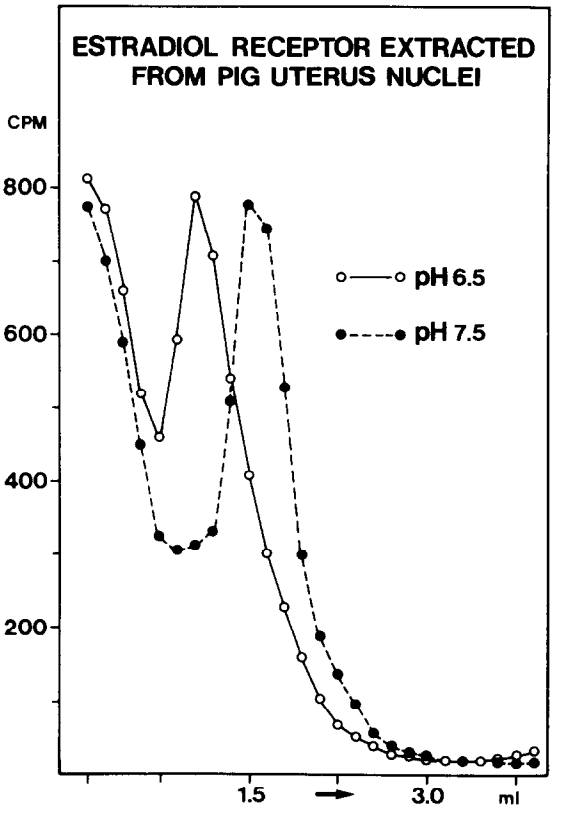


Fig. 3. Nuclear estradiol - receptor complexes. Stripped pig uterine nuclei extracted by sonication (3 × 3 bursts) and subsequent heating (30° × 30 min) in a 0.01 M phosphate buffer pH 7.5 containing 0.3 M KCl, 0.05 M DTT, and labelled estradiol (6×10^{-8}M). Analysed on 5 - 20% linear sucrose gradients 13 h × 56.000 rev./min (SW 56, Beckman L2-65B) at pH 6.5 and 7.5. Constant vol. sampling by upward displacement.

Based on this evidence, we suggest that the attachment of estradiol to a receptor molecule induces a conformational change of the protein, exposing formerly hidden groups, which then allow for the formation of steroid receptor dimers. For steric reasons, a back to back and head to tail attachment of the two participating monomers is the most likely configuration of the dimer (Fig. 1). The dimer is 'nucleotropic' and is the active principle in the nucleus.

The biosynthesis of estradiol receptor

Exposure of estradiol-deprived target cells to a single dose of the hormone leads to a decrease in cytoplasmic receptor concentration and a subsequent rise, which often exceeds the starting level [10 - 12]. We have studied this depletion - replenishment cycle in ovariectomized rats and pigs after intrauterine injections *via* the cervix. The anatomy of the rat allows for injection into one horn only, while the second horn serves as a control. The injection requires anesthesia. The two horns of the pig uterus are linked to a small corpus uteri and a common cervix. Surgical detachment of one horn provides a

similar situation to that naturally existing in the rat. Pigs can be trained for the intracervical injection, which resembles insemination by the boar.

A typical response after injection of estradiol into

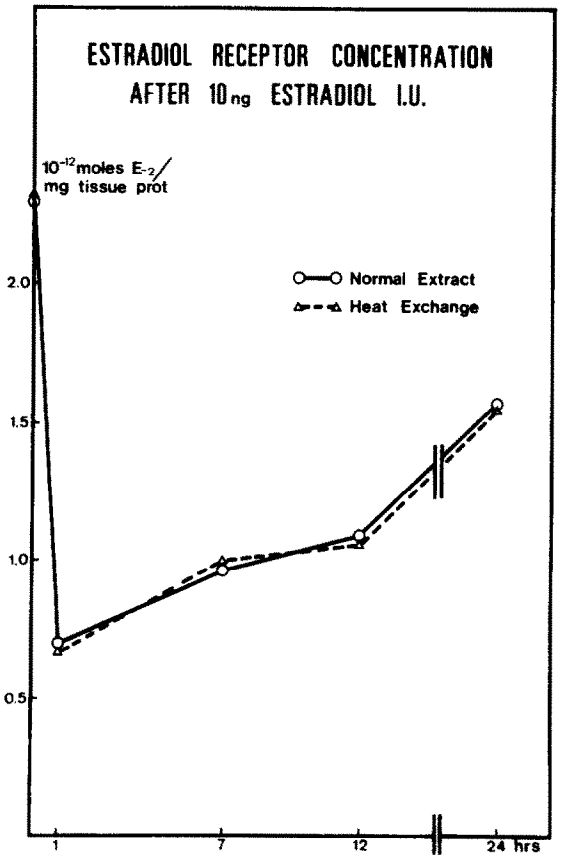


Fig. 4. Depletion - replenishment response of rat uterine cytosolic receptor to a single i.u. injection of estradiol.

the rat uterus is shown in Fig. 4. The full line represents the direct measurement of cytosol receptor concentration by incubation with labelled estradiol and subsequent analysis of the hormone receptor complex formed by agargel electrophoresis. For the dotted line, aliquots of the extracts were subjected to a heat-exchange procedure, i.e. pre-treatment with charcoal at low temperature, incubation with 6×10^{-8}M labelled estradiol for 30 min at 30° C, cooling to 0° C, charcoal treatment, than agargel electrophoretic analysis. This procedure would uncover the presence of unlabelled estradiol - receptor complexes left from the injected steroid [13]. Since both curves coincide, the estradiol receptor complexes formed in the cytoplasm must be rapidly transferred into the nucleus. The delay of the replenishment phase by i.u. administration of puromycin (Fig. 5) strongly indicates that it is caused by receptor synthesis and not by a recycling of receptors, from which the attached steroid has been released and channeled out of the cell by some unknown route. It appears therefore, that receptors

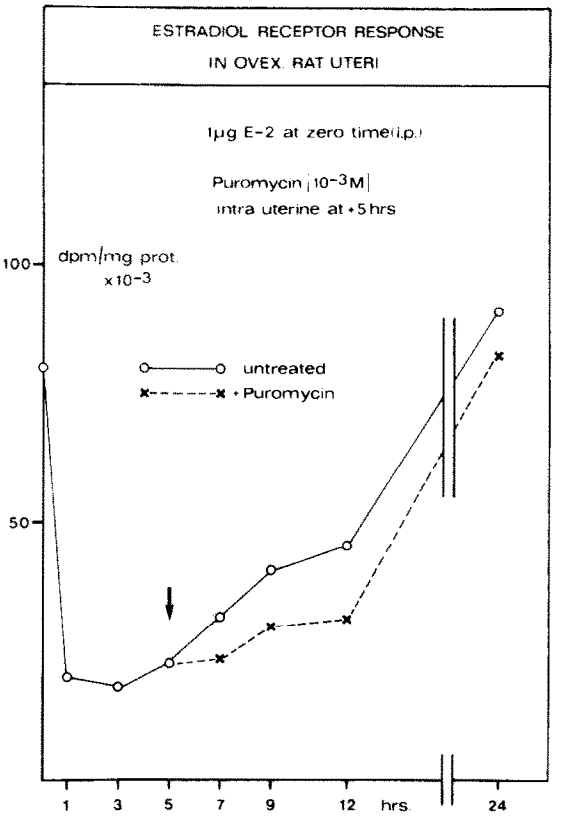


Fig. 5. Effect of puromycin i.u. on depletion - replenishment response.

are used only once and that the course of action includes a signal for receptor synthesis. This signal is the transcription of the receptor message, which can be inhibited by Actinomycin D (Fig. 6).

The subsequent translation starts betweeen 60 and 90 min after hormone administration as judged from the rise in microsomal receptor concentration observed in the experiments with pigs (Fig. 7). The rapid uptake of the hormone from the injected solu- [illegible] by the uterus cells and the quick drain of the excess hormone into the peripheral system where it is metabolized, allow for an accurate assessment of the retention time, which amounts to 2.5 h (Fig. 8).

The overall sequence of events is depicted in Fig. 9. It gives no indication of what happens to the steroid - receptor complex after its action in the nucleus. This important aspect is still unknown. The enhancement of receptor synthesis by the steroid-receptor: [illegible] dimer can thus be considered a highly specific phenomenon, occurring in all types of target cells, regardless of their state of differentiation, determining the range of products synthesized under hormonal influence.

Ovarian-independent fluctuations in estradiol receptor concentration

The form of the depletion - replenishment response elicited by estradiol depends on the starting level of the receptor concentration. We registered

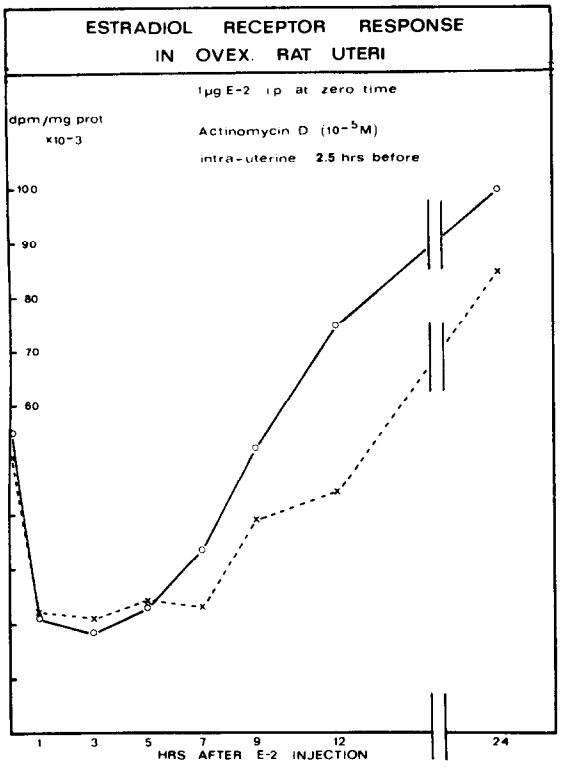


Fig. 6. Effect of Actinomycin D i.u. on depletion-replenishment response.

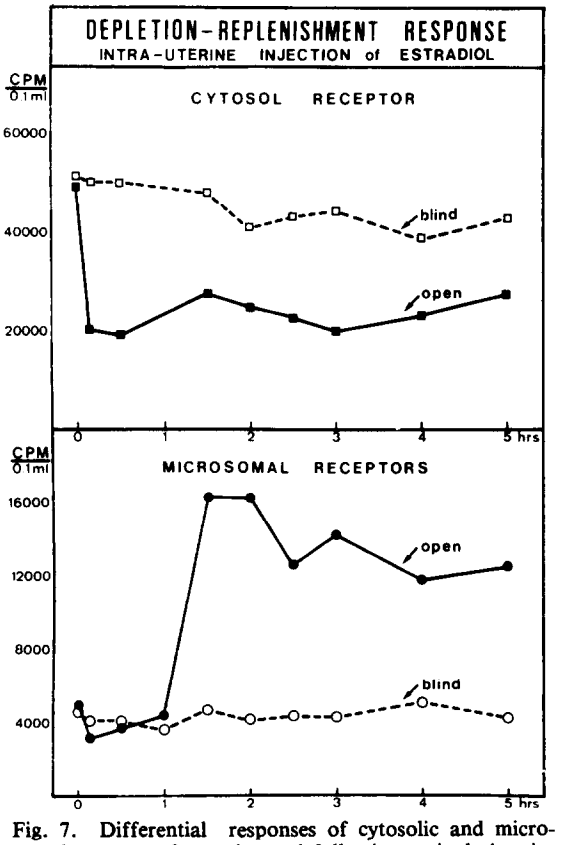


Fig. 7. Differential responses of cytosolic and microsomal receptors from pig uteri following a single i.u. injection of estradiol (2×10^{-6}M). Blind: control, uninjected horn; open: injected horn.

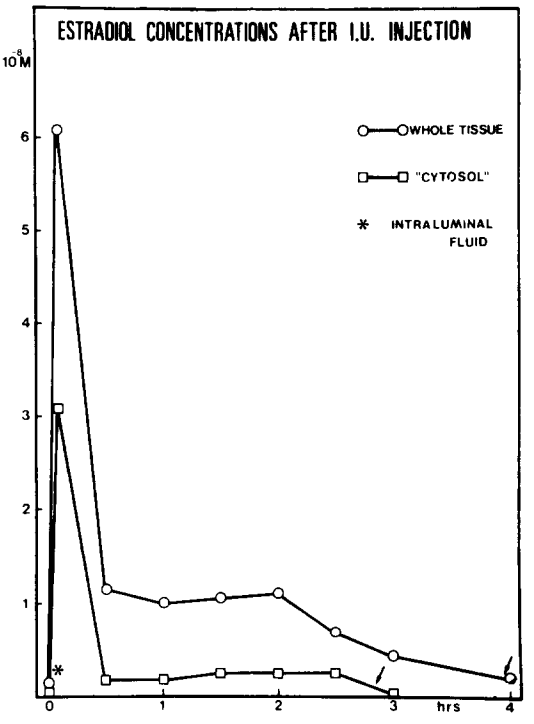


Fig. 8. Estradiol levels in pig uteri, following i.u. injection of estradiol (20 ml of a 2×10^{-6}M solution). Determinations by radioimmunoassay. Arrows indicate detection limits of assay.

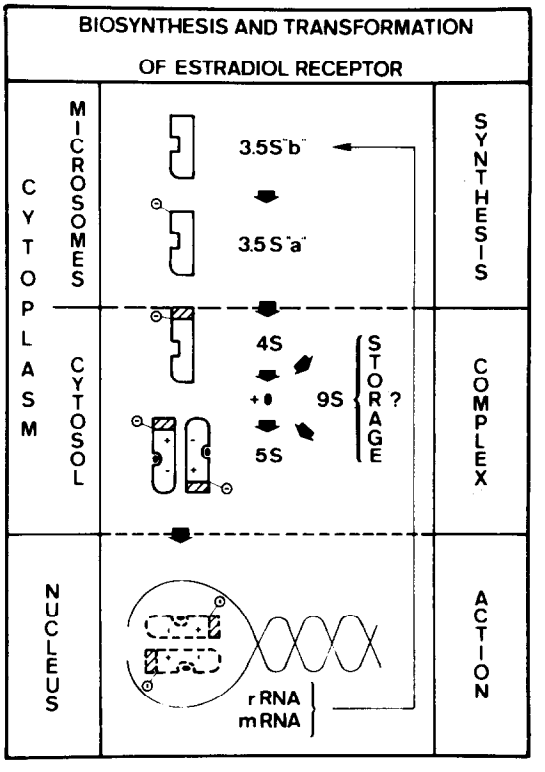


Fig. 9.

a seasonal variation in the receptor content of calf uteri, the uteri of ovariectomized pigs and in breast cancer biopsies from postmenopausal women [14]. The governing physical factor is more likely to be temperature rather than light. Pig uteri collected monthly over one year, the blind control horns from receptor biosynthesis experiments, had only a single, summer low in receptor concentration. The breast cancer specimens collected over five years in addition showed a 'central-heating' low when plotted by month

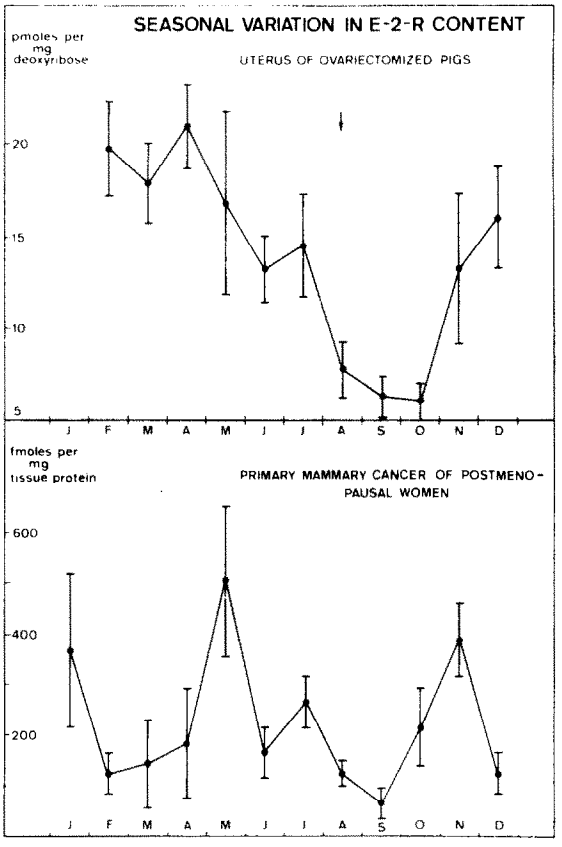


Fig. 10. Seasonal fluctuations of estrogen-receptor concentrations in uteri of overiectomized pigs (upper panel) and primary mammary cancers from postmenopausal women (lower panel). Arrow represents the temperature maximum for 1975.

(Fig. 10). Dr. Teulings [15], who employs our assay technique, arrived at virtually the same values for the breast cancer biopsies analyzed in Rotterdam. The biochemical steering mechanism is possibly a seasonal variation in the output of adrenal steroids, either estrogens or peripherally aromatised adrenal steroids [16]. These might also be responsible for the circadian rhythm of estradiol receptor concentration in the uteri of overiectomized rats [14].

Steroid-independent turnover of estradiol receptor

Even after the removal of ovaries and pituitary or of ovaries and adrenals, the uterine estradiol receptor concentration of rats does not maintain a steady level. It fluctuates with a period of some 3 - 10 days [8]. Receptor degradation and biosynthesis therefore must proceed even in the absence of estradiol and are thus basically hormone-independent processes. The first indication for an involvement of steroid-less receptors in the regulation of their own biosynthesis is given by the results of the following experiment: ovariectomized pigs were pretreated with estradiol by silastic tubing implants containing a crystalline suspension of the hormone. Eight days after the removal of the implant, the animals were slaughtered, a clean nuclear fraction was prepared from the uteri and the nuclei stripped of the outer layer of their envelope. The fraction was analysed for receptor and estradiol content. The concentration of receptor exceeded that of estradiol by a factor of 2.4. The number of estradiol molecules per nucleus (determined by radioimmunoassay) was 1270, whereas the total estradiol binding sites per nucleus was found to be 3010. Unless hormone and receptor can be released from the nucleus at different rates, the excess of receptor must have been accumulated in the nucleus without hormonal support. Since it was present in the 5*S* dimer form, dimerization can only be considered as hormone-facilitated but not as hormone-dependent. The presence of estradiol in the uterine nuclei of chronically ovariectomized pigs can again be explained by an adrenal production of estrogens or a peripheral aromatisation of other adrenal steroids.

Concluding remarks

The question whether steroid hormones initiate biological processes of only control their rate is not one of purely academic interest. A rate-controlling function can very well be the mechanism by which steroid hormones participate in cell differentiation. But what happens in cells released from this control, such as in cancer, where 'receptors' could continue to maintain the transcription of their own and other messages autonomously? In order to stop the growth of the cancer, it would then not only be necessary to completely (!) remove the activating steroids, but also to incapacitate the 'receptors'. The results presented by Dr. Lippman [17] surely lend weight to this concept and should, like our own results, encourage the search for selective receptor poisons.

Although we have been concentrating our efforts on the estradiol-receptor system, we believe that the principles found are also valid for the other steroid receptor systems. The progesterone receptor system has been extensively studied by O'Malley and associates [18]. They too assign the transcription-enhancing activity to a hormone-receptor dimer, both monomers of which are steroid binders, but differ in other physico-chemical properties. The mechanism they propose is a facilitation of RNA polymerase insertion by an interaction of the one monomer with DNA, while the other half of the dimer binds to an acidic protein. We proposed an unwinding effect of the highly symmetrical estradiol-receptor dimer by interaction with complementary structures arranged in opposite directions, meaning a direct interaction of both dimer constituents with DNA [4]. No matter which of the two proposals prevails, or even if both

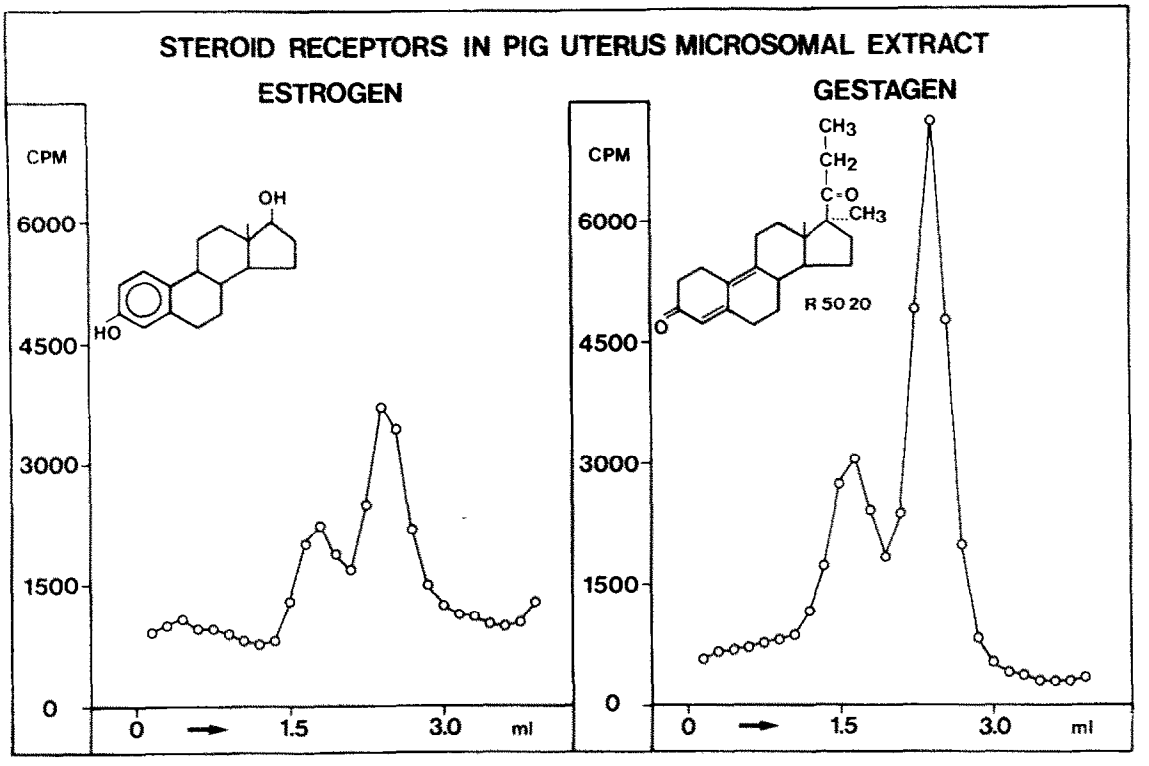
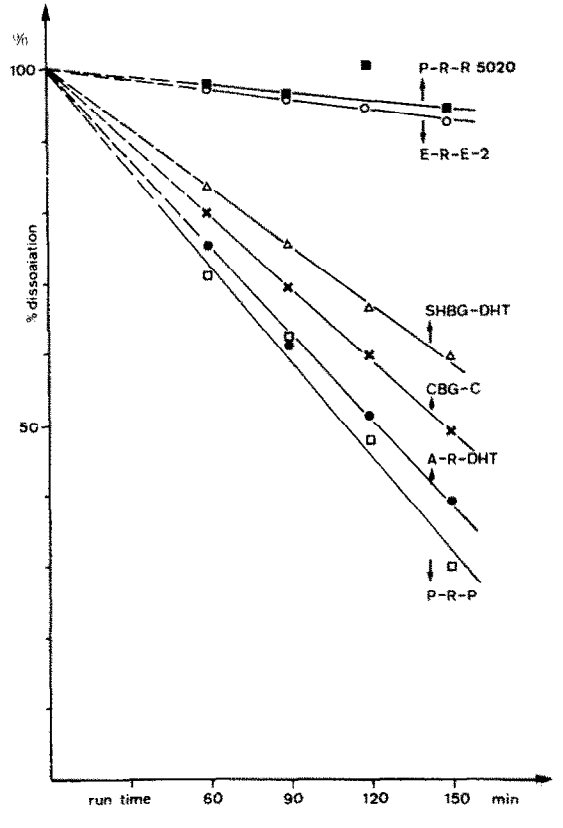


Fig. 11. Microsomal receptors for both estradiol (left side) and progestagen (right side). Analysis by sucrose density gradient centrifugation.



prove to be wrong, there is no doubt at all that the true mechanism will be a very specific one, since several steroid receptor systems exist alongside within the same cell [19, 20]. That all of them, like the estradiol receptor, follow depletion - replenishment cycles and that they are synthesized on cytoplasmic structures is a more than reasonable assumption. Using the superprogestagen R 5020, we have been able to ascertain our previous finding [4] of microsomal progesterone receptors (Fig. 11) and we hope that the synthetic androgen R 1881 will soon be available to verify the existence of androgen receptor precursors in the microsomal fraction of target cells [4, 21].

Fig. 12. Dissociation of various 'high affinity' complexes between steroid hormones and steroid binding proteins during analysis in agar electrophoresis.
P-R = progesterone receptor, E-R = estrogen-receptor, A-R = androgen-receptor, SHBG = sex hormone binding globulin,
CBG = corticosteroid binding globulin,
R 5020 = 17.21-dimethyl-19-nor-4.9-pregnadiene-3.20-dione,
E-2 = estradiol, DHT = dihydrotestosterone, C = cortisol.

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DISCUSSION

G. Rousseau. Concerning this estradiol-binding protein you find in uterus microsomes, did you check the binding specificity for non-estrogenic steroids. Secondly, to what extent is this microsome preparation devoid of constituents of nuclear origin?

Jungblut. Yes we did. The microsomal estradiol receptors are not identical with those for dihydrotestosterone and progesterone [4]. We could not detect DNA in the microsomal fractions, which consist of rough and smooth ER and other membrane fragments.

Kellie. Before the days of radioimmunoassay back in 1968, we, like many other people, used the rabbit uterine cytosol as a source of receptor protein for binding. Our experience was that during the summer months, we prepared the uterine cytosol and had no difficulty in finding a specific receptor. When however it came to the winter months, we killed many rabbits and prepared the cytosol by the same process but could find very little specific binding and a great deal of non specific binding. It was this primary observation that led us to develop the dextran-charcoal method of measuring estradiol receptors and we decided to apply this to an animal to find out whether there was some kind of seasonal variation. Now for our purpose the rabbit was unsuitable, (a) because it was expensive and (b) because it does not ovulate spontaneously, so we applied the receptor assay method to the rat uterus and we observed a variation throughout the estrus period low at estrus, high at diestrus and this cycled regularly, so it is not possible that this variation in cytosol receptor has something to do with the fertility cycle?

Jungblut. That might be quite possible. I am aware of the changes in uterine receptor content during the estrus cycle found in your and other laboratories. The seasonal and circadian fluctuations we observed were in immature or ovariectomized animals and postmenopausal women.

Kellie. Might I just ask whether in ovariectomized, hypophysectomized animals you tried the effect of administering estradiol?

Jungblut. Yes, we get the same depletion - replenishment response.

Kellie. And were receptors formed? There are many pointers that estradiol may in fact be a stimulus towards the formation of receptors. The fact that during the estrus cycle in rats the high blood levels precede increased formation of the receptor. That when you administer estradiol with a consequential decrease in the cytosol receptors there is a recovery phase during which estradiol receptors are again formed.

Junglbut. Yes, that's what we are trying to show.

Cidlowski. I'd like to ask one question about your replenishment studies in the pig uteri. A number of workers including Dr. Clark, Dr. Gorski and Dr. Muldoon have shown that significant replenishment occurs prior to 5 h. Is the 5 h replenishment of cytoplasmic receptors the earliest you see in the pig uteri?

Jungblut. The slide I showed is an old one, on which c.p.m./0.1 ml of extract are plotted over time. When instead the molar radio of receptor to DNA is used as the more appropriate parameter, we see that the replenishment phase of the cytosol receptor initiates only shortly after the rise in microsomal receptor concentration occurs. The reason for the misinterpretation from the old slide is the so-called 'water'-imbibition, which results in a weight-doubling of the trated born at 90 min after the i.u. injection of estradiol. Because of the admixture of extracellular proteins, the 'cytosol' protein concentration does not change and is also an unsuitable reference. Even the attempt to correct for the influx of plasma proteins by albumin assay fails in this extreme situation, because its proportion to the other plasma proteins is not known.

Cidlowski. Does cycloheximide block the microsomal replenishment?

Junglut. It is blocked by actinomycin D. Experiments with translation-inhibitors are schedules.

Clark. We have tried injections into the lumen of the uterus but we have had difficulty getting any effect from them. Now I noticed in your figures that the puromycin or cycloheximide only gave a partial block to the replenishment. How much did you have to use?

Jungblut. We injected 20 l of a 10^{-3} M solution of puromycin 5 h after estradiol administration. This delayed the replenishment phase until the puromycin was used up.

Clark. You don't use any special solution to get it in.

Jungblut. No, the trick is to make use of the increased permeability of the cell membrane during the 'water'-imbibition phase.