RIBONUCLEIC ACID SYNTHESIS
AND OESTRADIOL ACTION IN THE UTERUS

By

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ABSTRACT

Some aspects of the fate of oestradiol in uterine cells and of a proposed «cascade» mechanism of its activity are discussed. After a newly described step of entry of the hormone into the target cells, oestradiol is recognized by an intracellular transfer receptor system delivering it selectively to specific Non Histone Chromatin (NHC) protein of very feable abundance, provoking in turn (in a still unknown manner) the transcription of one (or a very few) gene(s). The resulting mRNA(s) would have a short half life and code for Key Intermediary Protein (KIP)(s) (also of very short half life). The latter activates the synthesis of essential component(s) of the cellular machinery in particular rRNAs, which are implicated post-transcriptionally in the generalized protein synthesis and therefore promote the growth of the differentiated cells. The reported changes in uterine RNA polymerase activity and protein synthesis are also discussed in the frame of other mechanisms.

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INTRODUCTION

Oestradiol is the physiological oestrogen found in all animal species examined. In mammals, its action has been best studied in the prepuberal or castrated rat atrophic uterus for which it is a growth promoting hormone. Uterine growth might be considered as a sort of amplification phenomenon. Most if not all chemical components of the atrophic, but nevertheless fully differentiated organ, are synthetized at increased rates under hormone influence, although differently in the various cell types of the uterus. The permanent hormonal dependence of the size, metabolism, etc., of the stimulated uterus, and conversely the reversibility indicated by the return toward the initial state when oestradiol is withdrawn, are basically different from the features of differentiation observed during embryonic development. It is consequently logical to look for a mechanism amplifying the expression of a pre-determined set of genes. A basic question is whether the switch of this mechanism will be found at the level of gene transcription or at any of the post transcriptional steps of the cellular machinery.

Within a few hours after the injection of a single »physiological« dose of oestradiol, there is ample direct and indirect evidence for increased protein synthesis in the uterus (see Fig. 1).

Fig. 1.
Alteration on uterine composition following a single dose of oestradiol (10 µg) injected to the rat at 0 time (after Mueller et al. 1958). RNA and protein are calculated as the ratio of uridine and protein to thymidine, respectively.
synthesis (Mueller et al. 1958, 1961), suppressible by puromycin or cycloheximide administered at the time of or just before the hormone administration. Whereas there is no increased DNA synthesis detectable before the 20th hour (Mueller et al. 1958; Billing et al. 1969a), there is an augmentation of RNAs preceding the bulk of protein synthesis (Fig. 1). Qualitatively, most of the newly formed RNA is ribosomal RNA (rRNA) when measured at 1–4 h after oestradiol injection, and an increase of all RNAs is observed at 3–5 h (Gorski et al. 1965; Hamilton 1968). The early increase of RNA biosynthesis plus the general understanding of the role of RNA in protein synthesis is compatible with the suppressive action of most anabolic effects of oestradiol by an inhibitor of RNA synthesis such as actinomycin D, administered just before the hormone (Ui & Mueller 1963)*. Therefore a simple and logical possibility is that oestradiol first increases directly and simultaneously («one step theory») the biosynthesis of all sorts of RNAs, which cannot be detected during the «lag» period because of the lack of sensitive techniques, but progressively become measurable when they are present in sufficient amount. These RNAs are in turn responsible for the increased synthesis of the enzymes necessary for the cumulative anabolic events.

$$O_{II} \rightarrow \text{RNAs} \uparrow \xrightarrow{\text{permissive proteins}} P \uparrow P \uparrow P \uparrow$$

It is of particular interest that inhibitors of protein synthesis such as puromycin or cycloheximide when administered just before or even immediately after oestradiol injection, abolish also the early increase in RNA synthesis (Mueller et al. 1961). There are several possible interpretations of such a finding. One of these, in the frame of the one step theory, predicts that the antibiotics block the synthesis of rapidly turning over permissive proteins implicated at some level in the processing and transport of the RNAs, in such a way that it prevents the oestrogen provoked RNA synthesis. In other words, it postulates that the inhibitors of protein synthesis abolish hormone action without blocking directly a component increased by the hormone. A radically different explanation, implicating a «cascade» theory (developed later and in Fig. 7) postulates the initial synthesis of a «KIP» (Key Intermediary Protein) protein, occurring during the first hour, sufficiently discrete, during this lag period, to be undetectable by the conventional techniques, but decisive for the development of most if not all of the macroscopic parameters of oestradiol action (possibly compatible with a mechanism discussion by Hershko et al. (1971)).

* It has been reported that part of the responses to oestradiol, such as nucleoside uptake (Billing et al. 1969b) or protein synthesis (Nicolette & Mueller 1966a), may withstand actinomycin D administered in vivo. It is difficult to ascertain whether or not it is due only to an incomplete access of the antimetabolic agent to the DNA sites or if there is an additional actinomycin D resistant mechanism of oestradiol action (possibly compatible with a mechanism discussion by Hershko et al. (1971)).
action occurring secondarily, including increased RNA synthesis (Baulieu et al. 1972). The use of actinomycin D could neither distinguish between the two theories, nor tell whether the first protein synthesized as postulated in the second theory is itself dependent on RNA synthesis and in the latter case, what kind of RNA.

\[
O_H \rightarrow \text{(RNA?)} \rightarrow \text{KIP} \uparrow \rightarrow \text{RNAs} \uparrow \rightarrow \text{P} \uparrow \text{P} \uparrow \text{P} \uparrow
\]

On the basis of this reasoning, two series of experiments have been undertaken, one dealing with RNA polymerase activity, which is the least poorly understood part of the RNA synthesis process, and the other looking systematically for RNA and protein synthesis during the first 60 min »lag« period after exposure to oestradiol.

**Oestrogen dependent increase of α-amanitin insensitive RNA polymerase activity and KIP**

At 1–3 h, there is a well documented augmentation of DNA dependent RNA polymerase (E. C.: 2.7.7.6) activity measured by the incubation of isolated uterine nuclei in Mg⁺⁺ 5 mM low salt buffer containing radioactive nucleoside triphosphate (Gorski 1964). This activity corresponds, at least in part, to nucleolar polymerase »A« or »I«, catalysing (pre-)rRNA synthesis (Kedinger et al. 1970; Blatti et al. 1970). The increased activity may be related not to a change of the »size« of the polymerase population but (to an increase of) the rate at which (it) can polymerize nucleotides into RNA» (Barry & Gorski 1971).

Only later, at 5–12 h, is there a discrete increase of the nuclear RNA polymerase activity measured in (NH₄)₂SO₄ (0.1–0.4 M) (Hamilton 1968; Blatti et al. 1970); it possibly corresponds to an extranucleolar enzyme »B« or »II« which catalyses DNA-like messenger? RNA (mRNA) (Widnell & Tata 1966; Kedinger et al. 1970).

The increase occurring 1–3 h after oestradiol administration to the rate of RNA polymerase A activity in isolated nuclei has been confirmed (Fig. 2). α-amanitin 10–100 nm (Fiume & Wieland 1970), added to the assay medium, reduces the production of radioactive RNA by exactly the same small amount in control and oestradiol treated uterus nuclei (Fig. 2, left panel) (Raynaud-Jammet et al. 1971). When RNA polymerase activity is measured in high salt buffer (Fig. 2), more radioactive RNA is obtained in both control and oestradiol treated preparations, and no difference is observed between the two groups. In presence of α-amanitin, the same amount of RNA is obtained in
Fig. 2.

RNA polymerase activities in isolated nuclei of oestradiol primed and control uteri. Uterine nuclei are prepared from homogeneous batch of immature control rats (C) or rats injected with 5 µg of oestradiol (OII). 3 h before sacrifice, and incubated for RNA synthesis according to Gorski (1964). Medium I is low salt and Mg++ 5 mM, and medium II is (NH₄)₂SO₄ 0.4 mM, Mg++ 5 mM. α-amanitin 10–100 µM (αn) is occasionally added. Each value is the mean of two determinations and four identical experiments gave similar results (Raynaud-Jammet et al. 1971).

Oestradiol treated (II OII+αn) and control (II C+αn) nuclei, and remarkably does not differ from that obtained in low salt control nuclei (I C+αn) but is lower than the value observed in low salt for oestrogen treated nuclei (I OII+αn).

These results cannot be definitively interpreted in absence of RNA analysis, actual measurement of enzymes, etc. . . and indeed superimposition of opposite effects of the high salt treatment may occur (Barker & Warren 1966). They have been discussed (Raynaud-Jammet et al. 1971) in terms of an oestradiol induced or activated factor, not functional in high salt, stimulating (pre)RNA synthesis, and which could be a different RNA polymerase (Chesterton &
Low salt RNA polymerase activity in isolated nuclei from surviving slices of oestradiol primed and control uteri.

Three hours after oestradiol or solvent injection, 4 slitted uterine horns from immature rats are incubated in Dulbecco modified Eagle medium at 37°C in 95% O₂ - 5% CO₂ atmosphere for various periods of time with occasionally cycloheximide (CHi, 25 µg/ml) or α-amanitin (αi, 1 µg/ml). Following the incubation, a nuclear fraction is obtained and the low salt RNA polymerase activity is assayed in medium I (see Fig. 2). Controls dealing with α-amanitin added only at the RNA polymerase assay step, have been performed and are marked an (Raynaud-Jammet et al. 1972).
Butterworth 1971; Smuckler & Tata 1971), or a polymerase subunit, or any other component of the chromatin machinery. Such a concept does not take into account the currently discussed changes of template availability to explain increased RNA synthesis (Barker & Warren 1966).

Indeed, there are experiments which suggest the necessity of protein synthesis to support the oestradiol induced increase of RNA polymerase activity. First puromycin and cycloheximide injected to the animal at the time of hormone injection, abolish the increase of the low salt RNA polymerase activity measured in oestrogen treated isolated nuclei (Gorski et al. 1965). In a second series of experiments, rats are injected with oestradiol, the uterus removed at 2-3 h, and uterine slices incubated at 37°C in Eagle's medium: a significant difference in activity of low salt RNA polymerase is maintained over a period of 2 h in favour of the oestrogenized over control nuclei (Nicollette & Mueller 1966b). Cycloheximide in the incubation medium of the slices abolishes the increment of RNA polymerase activity due to oestradiol (Nicollette & Mueller 1966b) (Fig. 3 O_{II} vs O_{II} + CHi), a result in favour of the necessary continued synthesis of an activating protein in the surviving slices. By changing the incubation time, an apparent half life of the protein factor can be approximatively determined, 15 min under these conditions (Raynaud-Jammet et al. 1972). Actinomycin D incubated with the slices blocks RNA polymerase activity in subsequently tested isolated nuclei, but one cannot conclude whether this is the consequence of the blockade of the end reaction (low salt RNA polymerase activity) or if it is because an initial RNA synthesis is necessary for the synthesis of the activating protein. However, α-amanitin in the slice incubation medium also abolishes the increase due to oestradiol of the low RNA polymerase activity (apparent half life of approx. 20 min) (Fig. 3 O_{II} vs O_{II} + a{i}). It has been seen (Fig. 2 left panel, O_{II} + a{n} vs C + a{n}) that α-amanitin does not block directly the already increased polymerase activity (see also in Fig. 3 the points indicated a{n}), and one, therefore, observes the inhibition by α-amanitin of the oestradiol induced increase of α-amanitin insensitive RNA polymerase.

Finally, these experiments can again be interpreted with the two different general view points. In the case of the »one step« theory, the increase of RNA polymerase A activity follows directly the interaction of the oestradiol-receptor complex with the gene transcription machinery, and the effect of cycloheximide and α-amanitin is due to the suppression of rapidly turning-over proteins (or their mRNA) which are not themselves increased by the hormone effect. Whether this or these »permissive« protein(s) are limiting for RNA polymerization, processing or transport (Church & McCarthy 1970) is not known. On the other hand, a »cascade« mechanism implicates that there is firstly an increased transcription of this rapidly turning-over protein implicated in the RNA increase which is then defined as KIP.
In any case, since no very early increased synthesis of the many mRNAs for the various proteins involved in tissue growth has been demonstrated, the augmentation in rRNA synthesis could be a mechanism for providing more protein synthesis through an amplification of the utilisation of these mRNAs.

»Induced protein« at 30 min and 15 S RNA at 15 min,
in vivo and in vitro

Thirty min after injection of oestradiol and using a gel electrophoresis technique an Induced Protein (IP) is visible (Notides & Gorski 1966). More recently two early protein peaks have been found (Mayol & Thayer 1970) and contrary to the first report, the suppressive action of actinomycin D has been demonstrated (Mayol & Thayer 1970; DeAngelo & Gorski 1970; Wira & Baulieu 1971), indicating a probable very early involvement of RNA synthesis in IP production.

Recent experiments in this (Wira & Baulieu 1971) and another (Katzenellenbogen & Gorski 1971) laboratory have apparently reproduced in vitro the IP obtained only until then in vivo. Uterine slices are incubated at 37°C in Krebs-Ringer bicarbonate glucose containing buffer for various periods of time with either [14C] leucine or [3H] leucine and oestradiol 1 nm. The tissues are pooled, homogenized in 0.05% sodium ethylene diaminetetraacetate, and the supernatant (15 000 × 6 g × min) is studied by electrophoresis on polyacrylamide gels. A series of controls indicates that the 3H/14C ratio of the various protein fractions might be taken as a good indicator of new protein synthesis. In Fig. 4 is indicated that there is electrophoretic coincidence between IP synthetized in vitro and in vivo after 1 hour exposure to oestadiol in the immature rat uterus, and that cycloheximide, actinomycin D and a-amanitin introduced in vitro with oestradiol at zero time have a suppressive effect. When actinomycin and a-amanitin are introduced 15 min after oestradiol, IP is obtained and this is compatible with its early occurrence (30 min incubation with castrated rat uteri: data not shown) and that of 15 S RNA (see later in this section and Fig. 6).

Instead of direct homogenization in the EDTA solution, a crude fractionation using sucrose has also been used to obtain fractions designated as sediment and cytosol, respectively. Gel electrophoresis reveals the same IP peak in both fractions when the extracts are obtained from uteri one hour after oestradiol injection and following 15 min in vitro incubation with radioactive leucine. However, the two IP do not survive for the same length of time in
slices subsequently incubated in the presence of a large excess of non-radioactive leucine. While the cytosol IP is still visible after 2 hours, the sediment IP disappears between 30 and 60 min. Therefore it is considered that the fractionation technique has separated two IP pools of different turnover (Wira & Baulieu 1972, unpublished).

A similar approach has been followed to provide evidence for the synthesis
Induced Protein (IP) in the cytosol and the sediment fractions of uterine homogenate. A time course study (Wira & Baulieu 1972, unpublished).

Rats are injected 1 h before sacrifice with either oestradiol or solvent, and the uteri incubated 15 min with $[^{3}H]$ and $[^{14}C]$ leucine, respectively. Thereafter, the incubation is continued for various periods of time in the presence of non-radioactive leucine (10 mg/ml). Crude supernatant and sediment fractions are first obtained after homogenization at low speed in 0.05% EDTA, sucrose 0.25 M. The $15000 \times 6 \times \text{min}$ supernatant is collected. The sediment is rehomogenized at high speed in 0.5 ml of 0.05% EDTA and the sediment extract is obtained after a $15000 \times 6 \times \text{min}$ centrifugation.

of discrete species of RNA. In these experiments, $[^{3}H]$ and $[^{14}C]$ uridine are incubated with uterine slices for 15 min. Homogenization is performed in sodium dodecyl sulphate 5%, acetate 0.1 M, Mg$^{++}$ 0.01 M, pH 4.5 buffer. RNA is extracted from the homogenate at 50°C with phenol and electrophoretized on polyacrylamide gels 15 min (Fig. 6). Up to 60 min after oestriadiol administration in vivo, in all cases, a 15 S species has been detected, and in many instances a minor peak (8 S) has been also observed. The 15 S RNA has also been observed after incubation in vitro with oestriadiol 1 nM for 15 min along with the radioactive nucleoside (Wira & Baulieu 1972a).
RNA from control and oestradiol stimulated uteri: a polyacrylamide gel electrophoresis (Wira & Baulieu 1972a). Some experimental details are in the text).

"One step" vs "cascade" theories: An evaluation

In summary, there is a sequence of events following exposure of the atrophic uterus to oestradiol in vivo or in vitro. By 15 min one observes 1 (or 2) RNA species, probably not ribosomal or transfer according to the size. Thereafter, at 30–60 min, appears a peak of IP corresponding to a limited number of protein(s), if not only one. This peak is probably due to some protein synthesis depending on RNA, the presence of which can be blocked by α-amanitin. It is possible to separate the two categories of IP and the one found in a crude nuclear fraction of the homogenate has a short half life (15–30 min). Finally,
Fig. 7.
Schematic representation of oestradiol action.
The upper graph is representative of the one step theory (a), whereas the 2 lower graphs describe a cascade mechanism (b) (c). b) refers to a sequence where only KIP synthesis is induced at the very beginning, and KIP = IP. On the contrary, in c), there is an increase of some other proteins parallelly to the initial increase of KIP, IP being some of them.


at 60 min and up to 120, there is an increase of RNA polymerase A activity which seems permanently dependent on the α-amanitin sensitive synthesis of a protein KIP of rapid turnover (half life 15–20 min).

As discussed before, all these data can be interpreted according to two main theories (Fig. 7).

In the »one step« theory (Fig. 7 a) there is no KIP but an immediate simultaneous increase of the various RNAs. The effect of cycloheximide and α-amanitin in the stimulated uterine slices on polymerase A activity is interpreted by an antagonistic effect on the permissive proteins for processing RNAs.

In the »cascade« theory, there is initial transcription of a KIP which activates RNA polymerase A activity. The nature of KIP is not understood and, among
other possibilities, it could be a protein leading to more RNA polymerase A like activity, or a factor engaged in the subsequent processing and/or transport of rRNA. The next step which relates the increase of rRNA synthesis to over all increased protein synthesis is also not understood in molecular terms.

Indeed, in the frame of the cascade theory, there are at least two possibilities. In one case, KIP is IP, following a rather simplistic (and likely overoptimistic) view . . . (Fig. 7 b). On the other hand, there is also the possibility that IP is not KIP but only one of the many proteins synthetized directly under the effect of oestradiol through a direct increase of their mRNAs which would be simultaneous with the KIP synthesis (Fig. 7 c).

**Oestradiol on its way to the RNA synthetizing machinery**

*Is the entry of oestrogen into uterine cells protein mediated?*  
(Milgrom et al. 1972)

It is usually admitted that oestradiol (and other steroid hormones), diffuse freely through the target cell plasma membrane. In this case, the first specific event in uterine cells would be the binding to the intracellular cytosol receptor protein (review in Baulieu et al. 1971). This concept has been challenged and, using α-iodoacetamide which inhibits the incorporation of radioactive oestradiol in the uterus after in vitro incubation of slitted horns (Jensen et al. 1967), an α-iodoacetamide sensitive step controlling the access of oestradiol into the target cells has been observed.

The in vitro incorporation of [3H] oestradiol in the rat uterus is measured following the suggestion of Williams & Gorski (1971): after tissue incubation with the radioactive steroid, an excess of unlabelled oestradiol is added, prior to homogenisation and bound radioactivity to the receptor is measured. This isotopic dilution prevents further undue binding of the radioactive steroid, whereas the previously bound radioactive oestradiol remains as such because of the very slow dissociation rate of the hormone receptor complexes at 0–4°C. The receptor bound radioactivity is measured in the cytosol and in the nuclear extracts and the sum is considered as the total incorporation of the steroid into the cells. The measurement of available receptor sites (»receptor content«) in the uterus is determined after an incubation without hormone under identical conditions as for the incorporation studies. Since no receptor is found in the nuclei when uteri have not been exposed to oestradiol, the cytosol is prepared and secondarily incubated with a saturating concentration of [3H] oestradiol and the receptor bound radioactivity is measured (Milgrom & Baulieu 1969).
Table 1.
Inhibition of radioactive oestradiol incorporation into uterine cells (Milgrom et al. 1972).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Radioactive oestradiol incorporation*</th>
<th>Receptor content*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>α-iodoacetamide 2 mM</td>
<td>4.7 ± 0.1</td>
<td>21.6 ± 1.5</td>
</tr>
<tr>
<td>α-iodoacetamide 10 mM</td>
<td>3.3 ± 1.3</td>
<td>23.5 ± 3.0</td>
</tr>
<tr>
<td>Iodoacetate 5 mM</td>
<td>25.9 ± 0.7</td>
<td>31.5 ± 1.9</td>
</tr>
<tr>
<td>Iodoacetate 30 mM</td>
<td>7.3 ± 0.1</td>
<td>33.6 ± 1.5</td>
</tr>
<tr>
<td>DTNB** 3 mM</td>
<td>74.6 ± 2.3</td>
<td>99.9 ± 9.5</td>
</tr>
<tr>
<td>DTNB** 10 mM</td>
<td>62.0 ± 3.5</td>
<td>89.7 ± 0.8</td>
</tr>
</tbody>
</table>

* Per cent of control with no inhibitor. ± SEM (3 experiments).

** Dithiobisnitrobenzoic acid.

Uterine horns are, at 37°C, preincubated for 10 min with the considered inhibitor and then incubated for 30 min in the presence of [3H] oestradiol 10 nM. Homogenization follows in buffer containing non-radioactive oestradiol 1 μM. Radioactive oestradiol bound to cytosol and nuclear receptors is measured. The receptor content is measured in the cytosol fraction after an identical incubation of uterine horns.

Preincubated with uteri, SH blocking agents decrease the oestradiol incorporation at 37°C as well as 25°C. The decreasing effect on the receptor content is always significantly smaller (P ≤ 0.01) than the decrease on incorporation. Time course experiments with α-iodoacetamide, especially at 25°C, confirm that the SH-blocking agent acts upon two different processes which do not evolve parallely. Preliminary measurement with parachloromercuriphenylsulphonate (which has limited penetration into the cells) are confirmatory. No specific effect on oestrogen entry has been observed with oligomycin, dinitrophenol or ouabain. It is therefore probable that some SH groups of protein play a direct or indirect role in the penetration of radioactive oestradiol into the target cells containing the receptor.

The saturability of oestradiol entry into uterine cells is observed when the incubation time is short (5 min) and when increasing concentrations of radioactive hormone are used. Calculations taking into account the affinity constant...
Oestradiol and diethylstilboestrol competition for binding to the receptor (a) and entry into cells (b) of [³H] oestradiol (Milgrom et al. 1972).

(a) [³H] oestradiol 0.5 nM is incubated at 37°C in the presence of increasing concentration of oestradiol and diethylstilboestrol with uterus cytosol. Bound radioactivity is measured.

(b) The entry of [³H] oestradiol 1 nM into uterine cells in the presence of various amounts of oestradiol and diethylstilboestrol is measured over a 5 min period.

(*B): Bound radioactive hormone concentration. (C): non-radioactive competitive hormone concentration. i: per cent of incorporated radioactive hormone in the presence of non-radioactive hormone (incorporation of radioactive hormone with non-radioactive hormone = 100 (3 experiments).
of the receptor, the number of sites and the small amount of oestradiol entering the cells over the incubation period, indicate that saturability is probably not due to receptor saturation but to a limitation of the entry. The latter shows some hormone specificity. Neither corticosterone, testosterone nor progesterone 1 µM depress the incorporation of radioactive oestradiol 10 nM. The decrease of oestradiol entry due to α-iodoacetamide is also observed for oestrone and oestriol. Finally it can be observed (Fig. 8) that, even though oestradiol and diethylstilboestrol compete for the incorporation of radioactive oestradiol, they do not do so equally (b), whereas they are equally bound to the solubilized receptor (a). Therefore, there might be a difference of hormonal specificity between the protein controlling the entry and the receptor protein.

All these results are subject to criticism because of the heterogeneity of the uterine tissue and the relative specificity of inhibitors. Moreover, entry into the cells is only one of a series of events concerned with the oestradiol distribution and the alteration of one of them can influence the other. Finally, since the measurement of entry is necessarily indirect because it is based on the secondary binding to the solubilized receptor, it would be necessary to exclude a different reactivity to SH-blocking agents and/or a different affinity for hormones of the receptor according to whether it is solubilized or inserted in some organized structure.

*The cytosol → neo-nuclear receptor* (see reviews in Raspé 1971)

In the cell, oestradiol is bound to an asymmetric highly hydrophobic protein, easily obtained in the cytosol fraction of the homogenate of castrated or immature animal uterus. The hormone induces a change of conformation of this protein, which provokes an operationally new localisation of the protein bound hormone in the cell. The complex appears now in the nucleus (Jensen et al. 1968; Gorski et al. 1968), from which it can be extracted by high ionic strength buffer. This is a justification of the »neo-nuclear« designation since there is no such binding protein in the nucleus before the arrival of oestradiol in the cell. The cytosol receptor oestradiol complex is then attached to a target organ specific nuclear structure, and recent experiments have designated as »acceptor« entities DNA itself and/or some acidic proteins of the chromatin.

At this point, it is pertinent to argue whether it is appropriate to give the very name of »receptor« to the receptor. The latter exhibits the necessary features which are hormone specificity, high affinity (K approx. 10^9-10 M^-1), target organ localization and limited number of binding sites per cell (some thousands). However, beyond its still unproven necessity for hormone action (even if it is probable), no »executive site« (Baulieu et al. 1971) has been demonstrated, and therefore the receptor may or may not be the last macro-

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molecule with which oestradiol interacts before the cellular response is triggered. In other words, the receptor described in this paragraph may not be the »real« receptor.

NHC protein (Alberga et al. 1971)
Among the nuclear Non Histone Chromatin (NHC) proteins of the immature uterus, one protein has the various features of specificity for oestradiol binding. It differs from the previously described receptor since it is already present in the nuclei of immature animal uterine cells and since it has a higher affinity ($\geq 10^{14}$ M$^{-1}$) and a smaller size. There are only a few molecules (approx. 10) per cell. Such a protein is a logical candidate for picking up a few oestradiol molecules released by the cytosol receptor imported into the nucleus (where there is likely no direct access to unbound oestradiol). No role can be ascribed to such a very high affinity chromatin protein at the present time but one may postulate a very rapid turnover of the protein itself: if not, the exceptional tightness of the hormone binding, which may even be covalent, is not compatible with a reversible regulatory mechanism. An alternative is a dramatic decrease of affinity when interacting with the appropriate chromatin component.

Action and receptors: an evaluation
There is indeed no demonstration that hormone transfer onto the NHC binding protein should follow the arrival of the hormone receptor complex from the cytosol. If this were the case, there would be numerical coincidence with the limited number of genes implicated as the origin of the cascade mechanism. On the contrary, the several hundred interactions realizable by the imported neo-nuclear receptor hormone complex would be more coherent with the picture designated as the one step theory (Fig. 7 a). Indeed, the two mechanisms may be simultaneously operative, as shown in Fig. 7 c, and possibly implicate two different types of oestrogen binding proteins as are the neo-nuclear receptor and the NHC protein.

It should be pointed out that all the uncertainties discussed here and elsewhere and concerning the localization and the role of the receptor proteins, remain unsolved. Moreover, the newly described initial interaction of oestradiol with the target cell, possibly at the membrane level, also evokes a possible control of precursor availability and for product segregation (Tata 1968), even if all results still indicate that the decisive switch is to be found at that level of transcription.

Cytosol receptor and RNA synthesis in in vitro subcellular systems
Before the work on RNA described above, this laboratory has been engaged in experiments designed to obtain an in vitro activation by oestradiol of the RNA synthetizing machinery and investigate the possible role of the receptor system.
The two questions have been partially answered (Raynaud-Jammet & Baulieu 1969).

When uteri are carefully homogenized in the constant presence of oestradiol 1 nm (physiological concentration), the nuclei subsequently isolated display more RNA polymerase A activity than when the same steps have been taken in the absence of hormone.

In other experiments, isolated nuclei are preincubated with either buffer or uterus cytosol, in the presence or not of oestradiol 1 nm. Thereafter the nuclei are reisolated by centrifugation and their RNA polymerase activity in a Mg++ low salt medium is tested. Only the preincubation with the intact cytosol plus oestradiol increases polymerase activity. These experiments have been recently reproduced and developed (Arnaud et al. 1971; Mohla et al. 1972); in particular the organ specificity of both the cytosol and the nuclei has been demonstrated, and also evidence has been presented that a specific conformational change of the cytosol receptor by oestradiol is a prerequisite for RNA polymerase (Mohla et al. 1972). Paradoxically, it is necessary to discuss the rapid and large increase of the low salt polymerase activity, and not the difficulty in obtaining reproducible results, because such an increase, easily reaching 200% in a few minutes, does not correspond to a similar augmentation of the polymerase A activity in vivo. Indeed, some very early (20 min) increase of Hn RNA (Hamilton et al. 1965) has been described and may correspond to an activation of polymerase B activity which could have been overlooked in vivo. Finally, it is not impossible that the in vitro reconstituted system of nuclei and hormone cytosol complex provides an interesting tool for studying some specific interaction of various pieces of the cellular machinery, even if these pieces are not necessarily organized as in vivo. In other words, even if some molecular details could be worked out with such a system, it might not reproduce the overall physiological mechanism.

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    661.


Raspe G. (ed.): Workshop on Steroid Hormone »Receptors«, Advances in the Bio-


Stancel: ... 
Baulieu: ... 
Wira: ... 

DISCUSSION

Erlanger: There are a great many synthetic progestins and oestrogens. How do their activities correlate with their binding activities in the in vitro systems?

Baulieu: It has been reported by many people, including in our group, that there is a sort of parallelism between affinity to intracellular binding proteins (measured at equilibrium and mostly at 4°C) of various natural and synthetic steroidal and non-steroidal oestrogens with their activity. However, the correlation is rather approximative, because there is no good quantitative in vitro system to test the oestrogen effect. When you inject these compounds into the animal and measure uterotrophic activity, for instance, you have to take into account transport, metabolism, and so on, before they get to the place where they act. The point I want to make is a little more interesting, and we are testing, not only for oestrogens, but for anti-oestrogens also, the components of the equilibrium constant, which are the association and dissociation rate constants. Those may vary in an unexpected manner and results could be the basis for further thinking about what to do in terms of antihormones.

Erlanger: Is this true also for progestins?

Baulieu: I don’t know. Dr. O’Malley can tell you that.

O’Malley: Yes, in our system protein induction by progestins correlates with their relative affinities for the receptor.

Ryan: Is what you are saying true for 19-norsteroids as well as derivatives of progesterone itself?

O’Malley: Yes.

Stancel: Dr. Katzenellenbogen has done some experiments with this induced protein, which is right now the only in vitro response we have got in the uterus, and you can correlate that reasonably well in terms of this induced protein caused by stimulation with different synthetic oestrogens. It appears to correlate fairly well with both the in vitro binding of the oestrogens to the receptor and also with the appearance, kinetically, of oestrogen receptor complex in the nucleus induced in vitro.

Dr. Baulieu, in your slide you showed that you could treat with oestradiol at time zero and at 15 minutes with actinomycin and apparently observe no decrease in the amount of induced protein. Dr. Katzenellenbogen has observed similar results. Can you shorten that time difference and still observe the induced protein?

Baulieu: We haven’t been earlier back to the oestradiol exposure time.

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Stancel: What is the time difference between the oestradiol and the actinomycin?

Baulieu: We haven't been earlier than 15 minutes between oestradiol and actinomycin or a-aminitin, or they have been given together.

O'Malley: It seems that for steroid hormones there is a general consensus of opinion that «facilitated», passive diffusion, and not really a transport process is responsible for hormone entrance into the cell.

Baulieu: Under the experimental conditions where incorporation is measured during a short period of time, inhibitors of metabolism have not done anything in that system to inactivate the transport.

Milgrom: There are three possibilities: either it is simple passive diffusion, or it is a protein mediated facilitated diffusion, or it is an active transport in which you have still the protein but you also need energy. We are inclined to think that oestrogen entry into uterine cells could correspond to the second possibility.

Ryan: One of the assumptions you make is that a-aminitin sensitivity has something to do with nucleolar versus non-nucleolar polymerase. How good is the evidence for this? I know you were asking Dr. Chambon. While we have the expert here, we should have a resolution of this question.

Baulieu: There is no good evidence that there is an a-aminitin insensitive polymerase in the nucleoplasm – that is all I know.

Chambon: I think, as I said in the discussion of my paper, that more work is required to prove that there is an a-aminitin insensitive RNA polymerase in the nucleoplasm of all animal cells.

Leroy: In spite of the impressive progress which has been accumulated in recent years in the field of sex steroid action, to people who are basically morphologists it appears somewhat frustrating that these data were obtained from studies on whole organs without making any difference between the various cell types of which target organs are composed. We know, as a matter of fact, namely from cell proliferation studies that in the uterus, for instance, muscle, endometrial stroma, glands and epithelium can react very differently to the same hormonal stimulus. Therefore we have attempted in collaboration with A. M. Preumont and P. Galand, to study transcriptional activity in different uterine tissues by using a method proposed a few years ago Brachet & Ficq (1965). It consists in revealing by autoradiography the labelled actinomycin-D (3H-AMD) bound in vitro by previously fixed histological material, so that one may qualify it as «Staining by actinomycin». There is now fairly well documented evidence that the amount of AMD bound to the nuclei is related to - let's say - the degree of chromatin activity. With the Chairman's permission I would like to present some of our data obtained with this method.

Fig. 1 shows that various hormonal treatments induce an increased AMD binding in both endometrial epithelium and stroma but that the pattern of response is different for each tissue.

In Fig. 2 one can see that the binding of [3H] AMD is increased by fixing with ethanol-acetic acid versus formalin, probably owing to the extraction of a good deal of the histones by the first fixative, as verified by Fast-Green staining. Nevertheless, the difference between controls (C) and 17β-oestradiol (E2) treated animals remains the same in both cases.
The third graph shows that oestradiol very rapidly augments AMD binding in the uterine epithelium and that a second substantial increase of binding occurs after a few hours lag period of hormone action. To our mind this two step evolution would be consistent with a cascade mechanism, as proposed by Dr. Baulieu in his talk.

These data are of course preliminary, but with respect to the idea that steroid action should also be studied at the level of individual cell types, I think that this kind of work is worthwhile to be pursued.
Fig. 3.

_Baulieu_: I would like to comment on that interesting set of experiments. What I don't know is how grains relate to the actual number of binding sites for actinomycin which are made available by oestrogen. I suspect, even though I don't really understand the technicalities of these experiments, that there are many binding sites, some of them only becoming visible. In that case, how does it fit with a limited number of newly synthesized proteins at the very beginning of hormone action, even we cannot say that there is only one protein (IP) for various reasons, as the soluble proteins which we extract may not be the only ones which are increased initially. The possible contradiction also raises a point which could be of great interest: maybe there are other aspects of initial oestrogen action, which appear at the same time, and are not expressed in terms of protein synthesis.

Now, conversely, we did experiments a few years ago together with Claude Raynaud-Jammet, which dealt with the possibility to involve directly the RNA nuclear synthesis _in vitro_ in isolated uterine nuclei in front of either oestradiol alone or the complex of oestradiol-receptor. The results were published (Raynaud-Jammet & Baulieu 1969) and recently reproduced by several laboratories. There is no increase of radioactive triphosphonucleoside incorporation into RNA but only when nuclei are exposed to the hormone cytosol complex. There is no increase when there is only oestradiol or cytosol, or if the cytosol proteins have been altered by previous heating.

When there is increase of RNA synthesis by the cytosol hormone complex, it is more than 300% of the background level, which is by far more than we can detect _in vivo_, and here is a contradiction. We, and also Jensen and others, have worked up more that system and showed that there is hormone specificity. So we are facing a very interesting problem, which is that maybe this _in vitro_ system is good enough to show some molecular specificity really involved in oestrogen action, but at the same time that it does not reproduce necessarily the physiological _in vivo_ response, as if the _in vitro_ situation had artificially opened up something.
Leroy: I only want to point out that in the results I showed, the oestrogen is given \textit{in vivo}, but the actinomycin binding is made on fixed material in order to eliminate the possible bias of permeability modifications by the hormone.

Milgrom: I would like to say something in support of Dr. Leroy's remark about the heterogeneity of the cells in the uterus. Miss Warembourg in Lille has studied by autoradiography, using a technique similar to that of Stumpf, the binding of progesterone in the guinea pig uterus. She has found a very remarkable heterogeneity in the fixation (Warembourg & Milgrom 1971).

Martini: I want to go back to the entry of oestrogen into the cell. Can you prevent it by giving ouabain or other compounds that block the sodium pump?

Baulieu: Ouabain was ineffective.

References: