The Transformation of the Cytoplasmic Oestradiol–Receptor Complex into the Nuclear Complex in a Uterine Cell-Free System

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Experiments performed with a cell-free system in tris–EDTA buffer, pH 7.4, indicate that the high-speed supernatant fraction of the rat uterus contains all the factors necessary to transform the 8S cytoplasmic oestradiol–receptor complex to the nuclear complex. The transformation is temperature-dependent. This nuclear complex was extracted in the form of a 5S particle with 0.4M-KCl from sediments of either uterine or heart nuclei that had been incubated together with the cytoplasmic soluble fraction of the uterus at 2°C for 30 min. This complex can also be obtained similarly from the soluble fraction of the uterus, incubated in the absence of nuclei. Previous warming of the soluble fraction to 37°C for 7 min was necessary for the successful extraction of the nuclear particle under these conditions of incubation. After an incubation of the transformed complex with the nuclear sediment at 37°C for 7 min, the 5S complex was extractable from the uterine nuclear sediment but not from the heart nuclear sediment, which may indicate the tissue specificity of the nuclear acceptor sites for the transformed complex. The extracted uterine nuclear complex sediments in the 5S region, but whether it is the native complex or a subunit or other part of the native complex resulting from the extraction with salt is unknown.

It is now known that a specific receptor protein for oestradiol-17 β is present in the cytoplasm of the rat uterus (Talwar *et al.*, 1965; Toft & Gorski, 1966; Jensen *et al.*, 1967; Toft *et al.*, 1967). This hormone–receptor complex has a sedimentation coefficient of approximately 8S. Further, an oestradiol–receptor complex that sediments in the 5S region has been extracted from either the nuclei or the chromatin of this organ by use of a tris–EDTA buffer, pH 8.5, containing a high concentration of KCl (Jensen *et al.*, 1968; Shyamala & Gorski, 1969; Jensen *et al.*, 1969). Experiments performed *in vitro*, however, suggest that the occurrence of the nuclear receptor complex is dependent on a preceding transport of the cytoplasmic 8S complex into the nucleus (Jensen *et al.*, 1968; Rochefort & Baulieu, 1968; Shyamala & Gorski, 1969). Jensen *et al.* (1969) demonstrated that the nuclear receptor complex sediments more rapidly (approx. 5S) than the subunit (approx. 4S) released by the cytoplasmic receptor (approx. 8S) in the presence of high salt concentrations. There is disagreement in the literature about the sedimentation coefficients of this subunit and the nuclear complex (see Erdos, 1968; Korenman & Rao, 1968; Puca *et al.*, 1971). It remains uncertain whether the nuclear particle extracted is the native complex or a subunit or other part of the nuclear complex released by extraction with KCl. Nevertheless, the occurrence of a particle sedimenting more rapidly (i.e. approx. 5S) than the cytoplasmic subunit serves as a good

criterion for the specific uptake of oestradiol by nuclear sediment in the cell-free system (Jensen *et al.*, 1968, 1969; Musliner *et al.*, 1970).

Jensen *et al.* (1968, 1969) demonstrated that the 5S particle can be extracted from the nuclear sediment of the uterus, after incubation at 25°C of the sediment together with [³H]oestradiol and cytosol prepared from this organ. This particle was not extractable from uterine nuclear sediment after incubation with oestradiol alone, or from nuclear sediment incubated with cytosol in which the cytoplasmic receptor had been previously destroyed by heating to 45°C for 30 min. Jensen *et al.* (1968, 1969) also reported that another particle, smaller than 4S, was extractable from the nuclear sediment of a non-target tissue incubated with the uterine cytosol containing the 8S [³H]oestradiol–receptor complex. The specific binding of oestradiol to the uterine nuclear sediment in the cell-free system, which yields a 5S complex on extraction with KCl, can thus be considered as one line of evidence that these analyses *in vitro* reflect to a significant extent reactions which occur *in vivo*.

We now report experiments which indicate for a cell-free system in tris–EDTA buffer, pH 7.4, that the cytoplasmic fraction of the uterus has the capacity to transform the 8S oestradiol–receptor complex into the nuclear complex, and that this transformation is temperature-dependent. Although uterine nuclei seem not to participate in this transformation, they

possess tissue-specific acceptor sites for the transformed complex.

Materials and Methods

Tissue collection and homogenization

Uteri or heart fragments taken from immature female Sprague-Dawley rats obtained from Hormone Assay Laboratories Inc. (Chicago, Ill., U.S.A.) and killed at 26 days after birth, were collected separately and used as indicated in the relevant Figures. In most experiments, uteri or heart fragments were removed from ten animals, pooled and washed in 0.9% NaCl. Uterine or heart tissue was minced finely with curved scissors for 10 min, and homogenized by use of a glass-Teflon homogenizer in 1 ml of tris-EDTA buffer (0.01 M-tris, 0.0015 M-EDTA, pH 7.4). The homogenate was separated into a nuclear sediment and a cytosol fraction by centrifugation at $150000g_{av}$ for 1 h at 2°C in a Spinco model L2-65B ultracentrifuge. In the preparation of nuclear sediment from heart, 300–350 mg of heart fragments, equivalent to the weight of 10 uteri, were taken from the same animals, and fractionated in the same manner as for the uteri.

Incubation and sucrose-gradient analyses

Uterine cytosol was incubated at 2°C for 30 min with 0.57 ng or 2.1 nmol of [2,4,6,7- 3H]oestradiol-17 β (95 Ci/mmol), obtained from New England Nuclear Corp. (Boston, Mass., U.S.A.). In several experiments, the cytosol was further incubated at 37°C for 7 min and re-cooled. A portion (0.5 ml) of the cytosol now containing the [3H]oestradiol-receptor complex was then incubated at the indicated temperatures and for the indicated periods of time with nuclear sediments obtained either from uteri or heart. After incubation, the reaction mixture was diluted with 5 ml of the tris-EDTA buffer, pH 7.4, and centrifuged at $200000g_{av}$ for 1 h at 2°C. The nuclear sediment that pelleted was resuspended in 0.8 ml of 0.4 M-KCl in the tris-EDTA buffer, pH 8.5. After centrifugation at $150000g_{av}$ for 30 min at 2°C, 0.25 ml of the supernatant fraction (i.e. the KCl extract) was layered on a sucrose gradient (5–20%, w/v) containing 0.4 M-KCl in the tris-EDTA buffer, pH 8.5. The gradient was centrifuged at $200000g_{av}$ for 18 h at 2°C. Bovine plasma albumin obtained from Calbiochem (Los Angeles, Calif., U.S.A.) was used regularly as a 4.7S marker (Jensen *et al.*, 1969) under these conditions of gradient analysis. In several experiments, cytosol was incubated at 2°C with [3H]oestradiol as described, then was made 0.4 M with respect to KCl, and was analysed similarly on sucrose gradients. This treatment of the cytosol with KCl is known to cause a release of a subunit (approx. 4S) from the approx.

8S [3H]oestradiol-receptor complex (Jensen *et al.*, 1969). As noted above, there is disagreement about the precise sedimentation coefficient of this subunit. We emphasize that we used the subunit not to determine the exact sedimentation coefficients of the several particles extracted in our experiments, but rather as an approximate marker to show that the nuclear particle sediments more rapidly (approx. 5S) than the subunit of the cytoplasmic [3H]oestradiol-receptor complex (cf. Jensen *et al.*, 1971). By use of an ISCO density-gradient fractionator, fractions (approx. 0.2 ml each) were collected directly into scintillation vials. Each vial then received 0.2 ml of water and 10 ml of scintillation fluid [4 g of 2,5-diphenyloxazole, 0.05 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 100 ml of Beckman BioSolv in 1 litre of toluene] for measurement of 3H in a Beckman LS-250 liquid-scintillation spectrometer, with an efficiency of 43%.

Terminology

Following Jensen *et al.* (1968), we use the expression receptor for the protein of the uterine cytoplasm which binds oestradiol to form the 8S hormone-receptor complex. In the discussion of the binding of the hormone-receptor complex by nuclei we use the expression acceptor sites, following King *et al.* (1971) and Steggle *et al.* (1971).

Results and Discussion

In agreement with the results obtained by Jensen and co-workers (Jensen *et al.*, 1968, 1969), we extracted, with 0.4 M-KCl, a 5S complex from the uterine nuclear sediment after incubation at 37°C for 7 min with uterine cytosol preincubated to form the [3H]oestradiol-receptor complex (Fig. 1, curve *b*). We also observed after incubation of the same components at 2°C for 1 h that no 5S complex was extractable from the nuclear sediment. Instead, another particle that sediments in the region of 4S was observed (Fig. 1, curve *a*). Several laboratories (Noteboom & Gorski, 1965; Jensen *et al.*, 1967; Puca & Bresciani, 1968) have shown that this particle, which may be identical with the 4S subunit of the cytoplasmic complex, is not extractable from uterine nuclear sediments after experiments with the hormone *in vivo*. Still another particle, sedimenting in the region of 3–4S, is extractable from the nuclear sediments of non-target tissues after their incubations at 25° and 37°C, respectively, with uterine cytosol containing the hormone-receptor complex. This is evident for nuclear sediments obtained from the heart (Fig. 1, curve *d*) and the diaphragm (Jensen *et al.*, 1968, 1969; Musliner *et al.*, 1970). Hence it is likely that the two kinds of particles noted represent subunits or other parts of the cytoplasmic complex

Fig. 1. Sedimentation profiles of KCl extracts of uterine or heart nuclear sediments after their indicated incubations with uterine cytosol containing the [^3H]oestradiol-receptor complex

The cytosol used was preincubated with [^3H]oestradiol at 2°C for 30 min to form the complex. The conditions of extraction with 0.4M-KCl, the use of bovine plasma albumin (BPA) and the 4S subunit (4S) as sedimentation markers, and the remaining details of experimental procedures were as indicated or as described in the Materials and Methods section. Curve *a* (●), profile occurring after incubation of uterine nuclear sediment and cytosol at 2°C for 1 h; curve *b* (○), profile occurring after incubation of uterine nuclear sediment and cytosol at 37°C for 7 min, and then at 2°C for 1 h; curve *c* (■), profile occurring after incubation at 2°C for 1 h of uterine nuclear sediment with uterine cytosol previously warmed to 37°C for 7 min; curve *d* (▲), profile occurring after incubation of heart nuclear sediment and uterine cytosol at 37°C for 7 min, and then at 2°C for 1 h; curve *e* (△), profile occurring after incubation at 2°C for 1 h of heart nuclear sediment with uterine cytosol previously warmed to 37°C for 7 min.

extracted by KCl, after a non-specific binding of the cytoplasmic complex to the nuclear sediment *in vitro*. In our experiments, the sedimentation profiles containing the 5S peak also usually show shoulders in the region of 3–4S (cf. Fig. 1, curves *b*, *c* and *e*), which again might be due to a non-specific binding occurring *in vitro*.

Our results (Fig. 1, curves *a* and *b*) indicate that warming of the uterine incubation mixture is necessary for extraction of the specific 5S particle from the uterine nuclear sediment. We observed, however, that this 5S particle is extractable after incubation at 2°C for 1 h of the uterine cytosol with uterine nuclear sediment, provided that the uterine

cytosol alone was previously warmed to 37°C for 7 min with [^3H]oestradiol (Fig. 1, curve *c*). Even after the incubation of this previously warmed uterine cytosol together with heart nuclear sediment at 2°C for 1 h, a 5S particle was extracted (Fig. 1, curve *e*). Hence it appeared that the uterine cytosol contains all the components required to transform the cytoplasmic oestradiol-receptor complex during warming to a particle that, after binding to nuclear sediment, yields the specific 5S particle on extraction with KCl.

To confirm this possibility, we incubated uterine cytosol in the absence of nuclear sediment, and adjusted it to 0.4M-KCl. Fig. 2 (curve *a*) shows that cytosol, preincubated at 2°C for 30 min to form the

Fig. 2. Sedimentation profiles of uterine cytosol preparations adjusted to 0.4M-KCl after their indicated incubations in the absence of nuclear sediment

The cytosol used was preincubated with [3 H]-oestradiol at 2°C for 30min. The remaining details of experimental procedures were as indicated or as described in the Materials and Methods section. Curve *a* (○), profile occurring after incubation of cytosol at 2°C for 7min; curve *b* (●), profile occurring after incubation of cytosol at 37°C for 7min. The total radioactivity under the profile of curve *a* was 34490 c.p.m., and that under curve *b* was 28310 c.p.m. 4S denotes the 4S subunit.

cytoplasmic [3 H]oestradiol-receptor complex, yielded the 4S subunit on adjustment to 0.4M-KCl, after a further incubation at 2°C for 7min. If, however, the preincubated cytosol was incubated at 37°C for 7min, treatment with KCl yielded both the 4S subunit and a more rapidly sedimenting particle occurring in the 5S region (Fig. 2, curve *b*). Whether this particle formed in the absence of nuclear sediment is truly identical to that formed in the presence of nuclei remains to be determined. Since this experiment was performed, Jensen *et al.* (1971) have reported a comparable experiment and drawn a similar conclusion.

Our observation that a 5S particle is also extractable from the nuclear sediment of heart tissue after incubation at 2°C (Fig. 1, curve *e*) raised the question of whether the cytoplasmic oestradiol-receptor

complex, once transformed, could bind to the nuclear acceptor sites of non-target tissues at the physiological temperature of 37°C. Fig. 3 (curves *a* and *b*) show that warming of the incubation mixture to 37°C for 7min, after an incubation at 2°C for 1h of the nuclear sediment with previously warmed uterine cytosol, caused the loss of most of the 5S particle extractable from heart nuclear sediment. No decrease in the amount of the extractable 5S particle was observed when uterine nuclear sediment was used (Fig. 3, curves *c* and *d*). This observation suggests, but does not prove, that at a temperature characteristic of cells *in vivo* only the nuclear sediment of the target tissue provides specific acceptor sites for the transformed complex. Stability of binding here could be a manifestation of tissue specificity for nuclear hormone-acceptor sites. We emphasize that Musliner *et al.* (1970) observed, with the same tris-EDTA buffer, pH 7.4, that the amount of the 5S particle extractable after the incubation of nuclear sediment and cytosol at 37°C for 6min is dependent on a previous heating of the nuclear sediment. Heating of the nuclear sediment to 60°C for 10min before the incubation with cytosol markedly decreased the amount of extractable 5S particle. Their interpretation was that the nuclear sediment contains a heat-sensitive factor required for the 'breakdown or processing of the 8S supernatant complex or the intranuclear binding of 4S and 5S complexes.' As our results described above indicate that uterine cytosol in the tris-EDTA buffer, pH 7.4, contains all the factors necessary for the transformation of the cytoplasmic complex to the nuclear complex, it seemed likely that heating to 60°C of the nuclear sediment destroys in some manner the specific nuclear acceptor sites for the receptor-hormone complex. Accordingly, we have determined that uterine nuclear sediment preheated to 65°C for 10min does not bind the transformed cytoplasmic particle during its incubation at 2°C for 1h with previously warmed cytosol.

We also examined the effect of warming uterine cytosol with [3 H]oestradiol on the sedimentation profile of the cytoplasmic oestradiol-receptor complex. Fig. 4 shows that after warming to 37°C for 7min, a relatively large amount of the receptor complex sediments to the bottom of the gradient in the form of aggregated particles. The total amount of radioactivity under the 4-5S peak, which may represent oestradiol bound to plasma proteins in the cytosol, is not significantly changed after the warming. The loss of radioactivity caused by heating in this experiment (Fig. 4, curve *b*; cf. Fig. 2, curve *b*) was also observed by Musliner *et al.* (1970) in related experiments. The loss is probably a consequence of non-specific binding of [3 H]oestradiol to the glass surface of the tubes employed, a heat-dependent process described by Clark & Gorski (1969).

Fig. 3. Sedimentation profiles of KCl extracts of uterine or heart nuclear sediments after their indicated incubations with uterine cytosol containing the [^3H]oestradiol-receptor complex

The uterine cytosol used was preincubated with [^3H]oestradiol-17 β at 2°C for 30 min, and then at 37°C for 7 min. Curve *a*, profile occurring after incubation of heart nuclear sediment and previously warmed uterine cytosol at 2°C for 1 h; curve *b*, profile occurring after incubation of heart nuclear sediment and previously warmed uterine cytosol at 2°C for 1 h, and then at 37°C for 7 min; curve *c*, profile occurring after incubation of uterine nuclear sediment and previously warmed uterine cytosol at 2°C for 1 h; curve *d*, profile occurring after incubation of uterine sediment and previously warmed uterine cytosol at 2°C for 1 h, and then at 37°C for 7 min. The total radioactivities under the profiles were as follows: curve *a*, 5305 c.p.m.; curve *b*, 3669 c.p.m.; curve *c*, 7714 c.p.m.; curve *d*, 6410 c.p.m. 4S denotes the 4S subunit.

The result of the preceding experiment raised the question of whether the 8S or the aggregated form of the warmed cytoplasmic hormone-receptor complex yields the 5S complex after binding to uterine nuclear sediment and extraction with KCl. We have recently observed that the aggregates are the active components of the warmed cytosol in yielding the 5S complex (M. Gschwendt & T. H. Hamilton, unpublished work). This suggests that warming of the cytosol results in a transformation of the cytoplasmic complex to a particle which aggregates *in vitro*, under the incubation conditions already described. However, the transformed particle *in vivo* may be transported to its nuclear acceptor sites very rapidly (see Williams & Gorski, 1971) and without aggregation. Even in the aggregated form, however, we find that the particle transformed *in vitro* is able to bind to the tissue-specific uterine nuclear acceptor sites *in vitro*, and yields the 5S complex upon extraction with KCl.

Our present results indicate that the cytoplasmic fraction of the uterus contains all the factors necessary to transform the cytoplasmic oestradiol-receptor complex to the nuclear complex. One possible problem here is that in the preparation of the cytosol

there is a transfer of nuclear factors to the cytoplasm as a consequence of the rupturing of some nuclei (cf. Jensen *et al.*, 1971). Nevertheless, our findings suggest the following sequence of events in the transport of oestradiol from cytoplasm to the nucleus in the uterus. The cytoplasm contains a specific receptor protein for oestradiol, which is not capable of migration into the nucleus. Binding of oestradiol by this receptor protein at physiologically significant temperatures alters its conformation and/or enables it to bind a transport-activating factor. This temperature-dependent transformation thus activates the oestradiol-receptor complex for transport across the nuclear membrane to interact with the tissue-specific nuclear acceptor sites, apparently chromosomal in location (King *et al.*, 1969; Steggle *et al.*, 1971).

Finally, we recognize that the relevance of the experiments with cell-free reactions of hormone-protein complexes *in vitro* to the reactions occurring *in vivo* remains to be understood fully, and note the current uncertainty about the characterization and sedimentation properties of oestradiol-receptor complexes isolated from the nuclear fraction of the uterus (cf. Vonderhaar *et al.*, 1970; Harris, 1971;

Fig. 4. Sedimentation profiles of uterine cytosol preparations after their incubations with [^3H]oestradiol

Uterine cytosol was prepared as described in the Materials and Methods section. After the indicated incubation, a portion (0.25 ml) of the cytosol was diluted with 2 vol. of the tris-EDTA buffer (pH 7.4), then was layered on a sucrose gradient (5–20%, w/v) prepared in the tris-EDTA buffer (pH 7.4), and was centrifuged at $100000g_{av.}$ for 18 h at 2°C in a Spinco model L ultracentrifuge. Curve *a* (●), profile occurring after incubation of the cytosol with [^3H]oestradiol at 2°C for 30 min; curve *b* (○), profile occurring after incubation of the cytosol with [^3H]oestradiol at 2°C for 30 min, and then at 37°C for 7 min. The total radioactivity under the profile of curve *a* was 33461 c.p.m., and that under curve *b* was 31354 c.p.m. BPA denotes bovine plasma albumin, ~8S denotes the approx. 8S complex and 4–5S denotes the 4–5S complex.

Puca *et al.*, 1971). This uncertainty notwithstanding, our ideas about the mechanism of transport of oestradiol from the cytoplasm to the nucleus in the

uterus are in broad agreement with the 'two-step' hypothesis of Jensen and co-workers (Jensen *et al.*, 1968).

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