*A TWO-STEP MECHANISM FOR THE INTERACTION OF ESTRADIOL WITH RAT UTERUS**

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During the past ten years much information has been accumulated concerning the interaction of tritiated estrogens with hormone-dependent tissues. Their striking affinity for estradiol, both *in vivo*[1-5] and *in vitro*,[6-9] first suggested that such target tissues as uterus, vagina, and anterior pituitary possess unique components called "estrogen receptors." Strong but reversible association of hormone with receptor, without chemical transformation of the steroid molecule, appears to be a primary step in the uterotrophic process, not affected by such inhibitors of early estrogen response as puromycin or actinomycin D.[10] The interaction of estradiol with target tissues involves two distinct phenomena—uptake and retention; the latter process, but not the former, becomes saturated *in vivo* if the hormone administered exceeds the physiological level.[11]

Centrifugal fractionation experiments[10-14] demonstrate two sites of estrogen binding in uterine cells. As confirmed by autoradiography,[15, 16] most estradiol, both in endometrium and in myometrium, resides in the nuclei, but a certain amount is bound to a macromolecular substance appearing in the supernatant fraction. Toft and Gorski[17] made the important observation that the radioactive estradiol-receptor complex in the supernatant fraction can be characterized by ultracentrifugation in sucrose density gradients where it migrates with a sedimentation coefficient of 9.5*S*; disruption of the complex by proteases but not by nucleases suggests that the receptor substance is a protein. We have confirmed these findings and have observed further[18-20] that a different estradiol-receptor complex, sedimenting at about 5*S*, can be extracted from uterine nuclei by cold 0.3 *M* KCl, which solubilizes little uterine DNA.[21] Although the 9.5*S* complex does not appear to be a simple aggregate of the 5*S*, the two receptor substances show many similar characteristics, discussed in more detail elsewhere.[22] An important difference is the ability of the 9.5*S* complex to form spontaneously on addition of tritiated estradiol to supernatant fraction;[19, 20, 23] the 5*S* complex is not produced by adding estradiol to nuclear extract,[24] although it can be formed in the whole homogenate.

This paper presents evidence that the 9.5*S* receptor is extranuclear and involved in estrogen uptake, whereas the nuclear 5*S* complex, probably responsible for hormone retention and growth initiation, is formed from the 9.5*S* complex by a process which consumes 9.5*S* receptor and which is retarded at low temperature.

Materials and Methods.—These investigations used estradiol-6, 7-H^3 (spec. act. 57.4 c/mmole), KRH buffer (pH 7.3, Krebs-Ringer-Henseleit-glucose, containing in gm/liter: NaCl, 8.0; KCl, 0.20; $Na_2HPO_4 \cdot 7H_2O$, 1.73; KH_2PO_4, 0.20; $CaCl_2$, 0.10; $MgCl_2$, 0.048; and glucose, 1.0), and Tris-EDTA buffer (pH 7.4, 0.01 *M* 2-amino-2-hydroxymethyl-1,3-propanediol plus 0.0015 *M* ethylenediaminetetraacetic acid, disodium salt).

For *in vivo* experiments, 23-day-old female Sprague Dawley rats were injected subcutaneously with estradiol-6, 7-H^3 in 0.9% saline–1% ethanol; for *in vitro* experiments,[16] excised horns were slit lengthwise and stirred magnetically at constant temperature in dilute solutions of hormone in KRH.

Uteri were homogenized in glass at 2° in 4 volumes of Tris-EDTA using seven homogenization periods of 15 sec, each followed by a 45-sec cooling period. The homogenate was separated into a supernatant fraction and total particulate sediment by centrifugation at 4° for 60 min at 204,000 × *g*. The sediment was extracted by gentle homogenization in cold 0.3 *M* KCl in Tris-EDTA. For density gradient analysis, 200-μl samples were layered on 4.8 ml cold, preformed 5–20% sucrose gradients in Tris-EDTA, which, for nuclear extracts, also contained 0.3 *M* KCl. After centrifugation at 4°, successive 100-μl fractions were displaced (by paraffin oil) into counting vials; as with homogenate fractions, tritium was determined directly in XDN fluor[22] at 27–30% efficiency.

The binding capacity of a supernatant fraction was determined by mixing a 200-μl portion with 50 μl of Tris-EDTA containing various amounts of tritiated estradiol; after standing 30 min at 2°, a 200-μl aliquot was analyzed on a sucrose gradient. In recombination experiments, the rinsed sediment from 26 pairs of uterine horns was resuspended by gentle homogenization in 3.3 ml cold Tris-EDTA buffer. Aliquot portions (0.8 ml) were mixed at 0° with solutions prepared by adding 0.2 ml of 1.7×10^{-8} *M* estradiol-6, 7-H^3 in Tris-EDTA to 0.8 ml of the three media described in Figure 3. After incubation, the three suspensions were chilled, centrifuged, and the resulting sediments extracted with 0.3 *M* KCl.

Results.—Ratio of supernatant to nuclear binding: When rat uteri, previously exposed to tritiated estradiol *in vivo* or at 37° *in vitro*, are homogenized in hypotonic Tris-EDTA, the supernatant fraction, which contains no significant radioactive component except 9.5*S* complex, represents 20 to 25 per cent of the uterine estradiol. If exposure to estradiol is carried out at 2° for a brief period, the

TABLE 1. *Uptake and distribution of estradiol-6,7-H^3 in rat uteri in vitro.*

Expt no.	Temp. (°C)	Estradiol ($\times 10^{-10}$ *M*)	Time (min)	Uptake*	Per cent in supernatant†
1	37	1	60	12.8	25
2	"	1	120	20.5	19
3*B*	"	2	30	15.1	22
3*C*	"	10	30	48.6	23
4	2	1	120	5.5	33
5	"	10	15	10.4	59
6	"	50	5	20.2	60
7	"	55	5	27.7	75
8*a*	2	55	5	21.9	69
8*b*		(Same as 8*a* plus standing for 15 min at 37°)‡			23
9*a*	2	69	5	27.8	66
9*b*		(Similar to 9*a* plus standing for 75 min at 2°)‡			54
9*c*		(Similar to 9*a* plus standing for 60 min at 37°)‡			20

* In femtomoles estradiol per mg wet tissue; maximum uptake after 0.1 μg injection *in vivo* is 15–20 fmole per mg.

† Shown by sucrose gradient ultracentrifugation to be essentially all 9.5*S* complex.

‡ Uteri removed from estradiol solution, washed in cold KRH, and incubated in a moist atmosphere before homogenization.

proportion of the total radioactivity present as 9.5*S* complex is markedly increased (Table 1). After such uteri are removed from the hormone solution, much of the 9.5*S* estradiol undergoes transfer to the nucleus. This redistribution is slow at 2° (expt. 9*b*), but at 37° the level of 9.5*S* complex falls to "normal" within 15

minutes (expt. 8). More than half of the 9.5*S* radioactivity which disappears during warming can be extracted from the nuclei in the form of 5*S* complex.

Localization of the 9.5S receptor: The question whether the 9.5*S* receptor is extranuclear or whether it is extracted from the nucleus during homogenization cannot be answered definitively by autoradiography of uteri in which the 9.5*S* complex comprises a small fraction of the total radioactivity. Because the extranuclear space greatly exceeds the nuclear volume, the concentration of such an extranuclear component may be too low to permit detection in the presence of the concentrated nuclear radioactivity. Uterine tissue with a high content of 9.5*S* complex, more favorable for autoradiographic localization of the 9.5*S* receptor, can be obtained after estradiol uptake at 2°.

Autoradiographs of uteri exposed to estradiol at 37° *in vitro* show nuclear concentration of radioactivity (Fig. 1*A*) similar to that observed *in vivo*.[15, 16] In contrast, uteri in which 60 per cent of the estradiol is present as 9.5*S* complex (expt. 6, Table 1) show a large amount of radioactivity outside the nuclei (Fig. 1*B*). The correlation of this extranuclear radioactivity with specific cytoplasmic or membranal components must await the application of staining techniques which delineate extranuclear cell structure.

Binding capacity of the 9.5S receptor: Because of the spontaneous formation of the 9.5*S* estradiol-receptor complex, the total 9.5*S* binding capacity of a supernatant fraction can be measured by adding to it increasing amounts of tritiated estradiol until the radioactivity of the 9.5*S* peak on subsequent gradient cen-

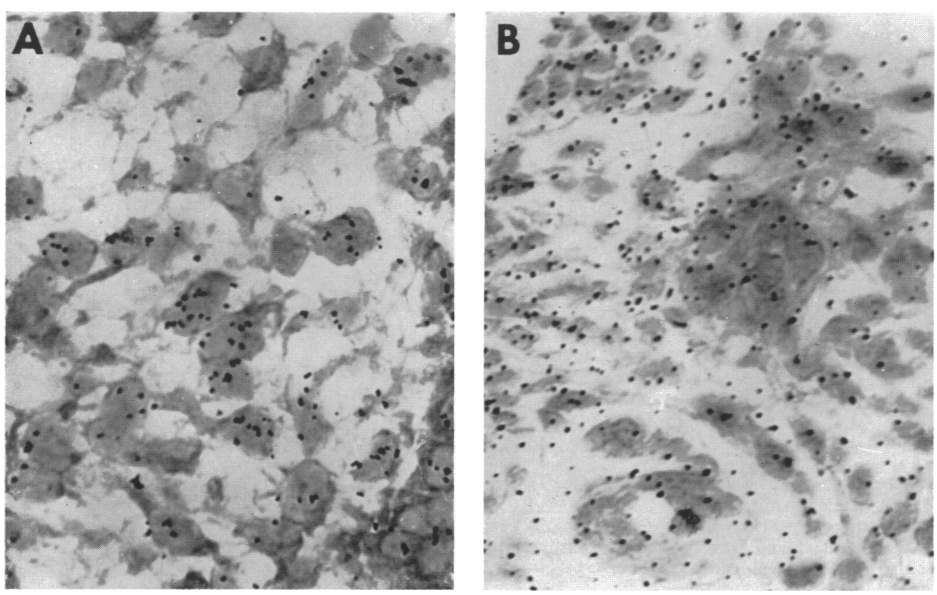

FIG. 1.—Autoradiographs, by procedure of Stumpf and Roth,[15] of 2-μ frozen sections of rat uterine horns after estradiol uptake *in vitro*. (*A*) Incubation at 37° for 60 min in 10^{-10} *M* estradiol-6,7-H^3; radioactivity in tissue, 8600 DPM/mg dry wt, 21% as 9.5*S* complex; autoradiographic exposure, 57 days. (*B*) Incubation at 2° for 5 min in 5×10^{-9} *M* hormone; radioactivity in tissue, 12,600 DPM/mg, 60% as 9.5*S* complex; autoradiographic exposure, 45 days.

trifugation no longer increases, and excess estradiol is evident.[20, 23] Such experiments reveal that even hyperphysiological doses of estradiol *in vivo* do not saturate the 9.5*S* receptors of rat uteri. Fifteen minutes after injection of 0.05 μg of tritiated estradiol, the 9.5*S* complex utilizes only 5 per cent of the binding capacity remaining in the supernatant; even with a 0.5-μg dose, only half the

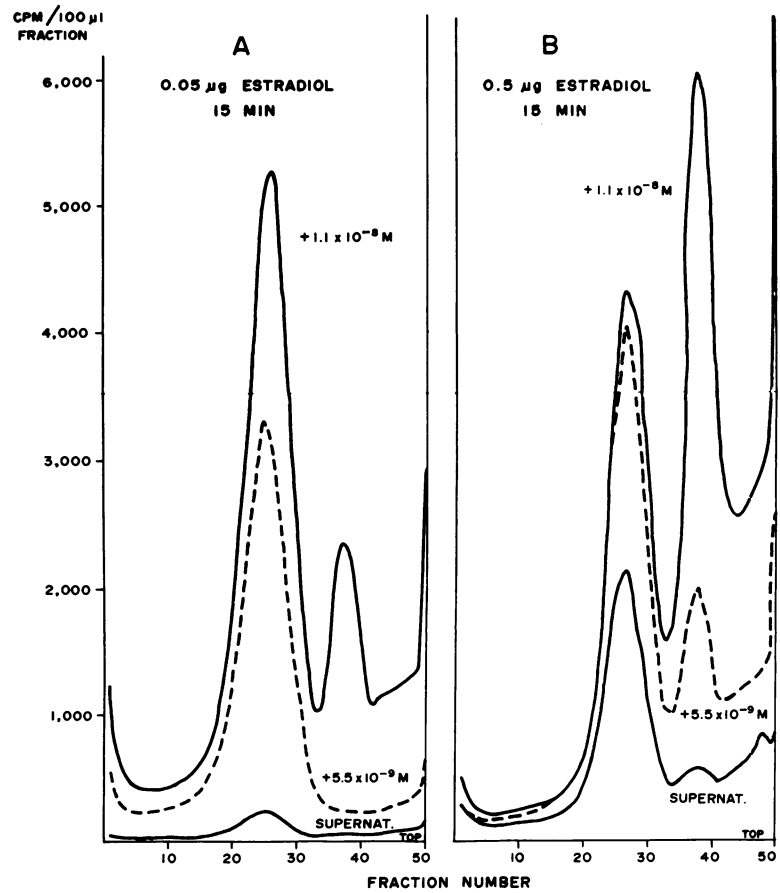

FIG. 2.—Sedimentation patterns in sucrose gradients of radioactivity in supernatant fractions, with and without added estradiol-6,7-H³, from rat uteri excised 15 min after injection of (*A*) 0.05 or (*B*) 0.5 μg estradiol-6,7-H³. Centrifugation: (*A*) 7 hr at 216,000 × *g*; (*B*) 7.3 hr at 204,000 × *g*. Molarity figures indicate total concentration of added estradiol in 250-μl system containing 200 μl of supernatant fraction. Because these uteri contain serum proteins, excess estradiol appears in the 5*S* region.[24]

9.5*S* receptors bear estradiol (Fig. 2). Similar lack of saturation was observed two hours after injecting 0.05 or 0.2 μg of hormone *in vivo* or after uptake of physiological or hyperphysiological amounts of estradiol *in vitro*.

In none of the foregoing experiments does the saturation value of the supernatant fraction represent the original level of 9.5*S* receptor in uterine tissue. This substance is rather labile and begins to disappear soon after the tissues are ex-

cised; moreover, exposure of uteri to estradiol depletes their 9.5*S* receptor content, as discussed below. By working rapidly at low temperature with uteri not previously treated with hormone, the extradiol-binding capacity of the supernatant fraction was found to be about 100 femtomoles per mg wet uterine tissue or about 2.1 picomoles per pair of horns. This value, which represents a 9.5*S* binding capacity double that of Figure 2*A* and more than twice that reported by Toft *et al.*,[23] undoubtedly is still lower than the actual level in the original tissue.

Depletion of 9.5S binding capacity during estradiol uptake: In addition to the lack of saturation of the 9.5*S* factor, Figure 2 also reveals that there is a significant

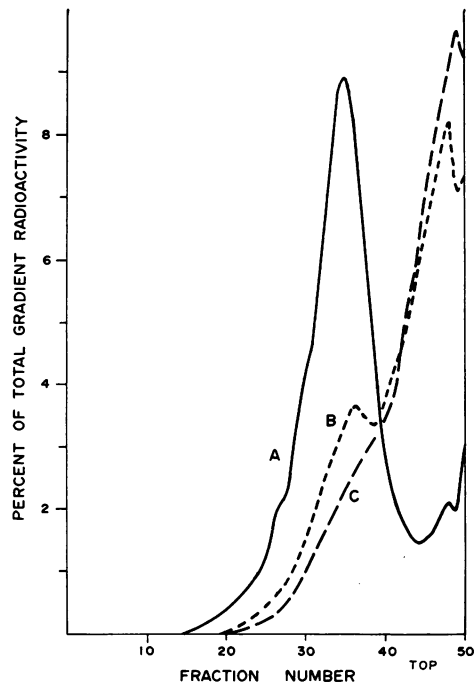

FIG. 3.—Sedimentation pattern in sucrose gradients of 0.3 *M* KCl extract of total particulate fraction of rat uterine homogenates after incubation at 37° for 5 min with 1.9×10^{-9} *M* estradiol-6,7-H^3 in: (*A*) supernatant fraction; (*B*) supernatant fraction previously warmed to 45° for 30 min; (*C*) Tris-EDTA buffer. Centrifugation for 10 hr at 216,000 × *g*. Total cpm in the gradients: (*A*) 2400; (*B*) 1940; (*C*) 2960.

reduction in total supernatant binding capacity as the dose of estradiol is increased. The areas under the peaks at saturation indicate that the supernatant fraction of uteri excised 15 minutes after a 0.5-μg dose of estradiol contains 13.8 femtomoles less binding capacity per mg wet tissue than it does after 0.05 μg of hormone. Similar depletion of 9.5*S* binding capacity with increased estradiol dose was observed two hours after injection of hormone *in vivo* and in uteri exposed to various concentrations of estradiol *in vitro*. In three out of four experiments, the decrease in 9.5*S* binding capacity corresponded closely to the increased amount of 5*S* complex which could be extracted from the nuclei. It would

appear that as more estradiol enters uterine nuclei to form 5S complex, the content of 9.5S receptor in the supernatant fraction is significantly lowered.

Requirement of a supernatant factor for 5S complex formation: Direct evidence for the participation of the supernatant fraction in the formation of the 5S estradiol-receptor complex is provided by experiments in which the particulate sediment from untreated uteri was incubated with: (*A*) supernatant fraction to which tritiated estradiol was added and shown to be present entirely as 9.5S complex; (*B*) estradiol in supernatant fraction which had been warmed to 45° for 30 minutes, destroying 9.5S binding; and (*C*) estradiol in Tris-EDTA buffer. During five minutes at 37° the sediments took up 66, 54, and 64 per cent, respectively, of the total radioactivity, about one third of which was solubilized in each case by a single extraction with 0.3 *M* KCl. Only in group *A*, in which the nuclei had been exposed to estradiol in the form of 9.5S complex, did the extract consist of 5S complex (Fig. 3). Similar results were obtained when the incubation of nuclear sediment with the three different estradiol-containing media was carried out at 10° for 30 minutes. It would appear that although uterine nuclei can take up and bind free estradiol, formation of 5S estradiol-receptor complex requires the presence of unheated supernatant fraction containing estradiol in the form of 9.5S complex.

Discussion.—The foregoing observations demonstrate that the uterine 9.5S factor participates both in estradiol uptake by uterine cells and in the formation of 5S estradiol-receptor complex in their nuclei. Autoradiographic studies of uteri, rich in radioactive 9.5S complex, show this factor to be extranuclear; the spontaneous formation of the 9.5S complex, even at 2°, indicates low activation energy for the uptake process. The high uterine content of 9.5S factor, not saturated even at hyperphysiological estradiol levels, explains the nonsaturable capacity of uterine tissue for initial estradiol uptake *in vivo*.[11]

Experiments with recombined homogenate fractions show that a heat-labile supernatant factor is required for production of 5S complex, suggesting that estradiol must be presented to the nucleus as 9.5S complex in order for 5S complex to be formed. In whole uterine horns, transfer of estradiol from a 9.5S to a 5S form takes place readily at 37° but slowly at 2°, suggesting that this process requires activation energy and possibly may be enzymatic. As estradiol is taken up by the nucleus, either *in vivo* or *in vitro*, 9.5S binding capacity disappears, indicating that the 9.5S receptor either is destroyed as it delivers estradiol to the nucleus or else that it actually is transformed into 5S receptor by loss of a molecular fragment. Investigations to determine whether uterine nuclei contain an enzyme catalyzing the selective degradation of the 9.5S complex are presently underway.

Summary.—The interaction of estradiol with uterine tissue appears to involve a two-stage mechanism. The hormone associates spontaneously with an extranuclear "uptake" receptor to form a 9.5S complex, a phenomenon which utilizes only a small portion of the total amount of this receptor available. The 9.5S complex transfers estradiol to the nucleus by a temperature-dependent process which consumes 9.5S receptor. Formation of the characteristic 5S complex by the nucleus requires the presence of supernatant fraction containing estradiol in

the form of 9.5S complex. It is suggested that 9.5S complex may be transformed into nuclear 5S complex by cleavage of the 9.5S receptor molecule.

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[24] If serum proteins are present, addition of tritiated estradiol forms a relatively weak complex, sedimenting at about 5S, which can be distinguished from the uterine 5S hormone-receptor complex by its lack of sensitivity to organic mercurials.[18]