4B, or 6B both suggest the presence of active particles of several molecular sizes. All attempts to break up the active particle by removing lipid resulted in complete destruction of the enzyme activities, which were not restored by the addition to the lipid-free extracts of micellar acetone extracts of microsomes.

At present, complete separation of the steroid glucuronyl and *N*-acetylglucosaminyl transferases with retention of both activities has not been achieved. However, the transferases can be selectively inhibited or destroyed in the solubilized microsomal extracts, and these procedures, together with filtration on Sepharose 2B, can be used to obtain partially purified preparations of either transferase activity essentially devoid of the other.

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Control of Estrogen Binding Protein Concentration under Basal Conditions and after Estrogen Administration*

Mary Sarff† and Jack Gorski‡

ABSTRACT: The control of estrogen receptor concentration in the immature rat uterus was studied under basal conditions (without estrogen), and after estrogen treatment. An estimate of the turnover rate of the cytoplasmic estrogen receptor was determined using cycloheximide to block protein synthesis. At various times after exposure to the inhibitor, the estrogen binding was assayed. When protein synthesis had been blocked 8–12 hr either *in vivo* or *in vitro*, the total binding capacity decreased only 6%. The turnover rate of the binding protein (estimated half-life of 5–6 days) was much slower than the half-life of 20–22 hr previously reported for the proteins of uterine cytosol. After a single intraperitoneal injection of 0.1 μg of 17β-estradiol, the estrogen binding capacity of the cytoplasmic estrogen receptor decreases by 50%. The binding capacity is replenished beginning about 6 hr after the estrogen injection, and by 16 hr the estrogen receptor level reaches control values. Cycloheximide or actinomycin D, when administered shortly before or 2 hr after estrogen, inhibits the return of estrogen binding in the cytosol. Cycloehximide or actinomycin D administered *in vivo* 6 hr after estrogen has little or no effect on the level of estrogen receptor binding. The data indicate that binding capacity is replenished at a time when synthesis of the binding protein does not occur. It also appears that both protein synthesis and RNA synthesis are involved in an early event that is essential for the replenishment of the estrogen binding protein seen after an *in vivo* surge of estrogen.

When 17β-estradiol is injected into immature or ovariectomized rats, it is selectively taken up and bound in tissues which are responsive to estrogens, such as the uterus, vagina, mammary glands, and pituitary gland. This fact has led to intensive investigations concerning the entity responsible for this binding, the so-called estrogen receptor (Jensen and Jacobson, 1962; Noteboom and Gorski, 1965; Toft and Gorski, 1966; Shyamala and Gorski, 1969). The 105,000*g* supernatant fraction (cytosol) of uterine cells contains a factor which specifically binds estrogens, *in vivo* or *in vitro*, and which sediments in a linear sucrose density gradient at about 8 S (Toft *et al.*, 1967; Erdos, 1968). Studies of this receptor's binding specificity, its size (mol wt ~200,000), and its sensitivity to proteolytic enzymes suggest that it is a protein (Noteboom and Gorski, 1965; Toft and Gorski, 1966).

The estrogen "receptor" concentration in the cytosol remains fairly constant at a calculated value of 16,000 binding sites/cell in the immature rat (Clark and Gorski, 1970) and in the ovariectomized rat (Notides, 1970). After estrogen injection, the cytosol receptor numbers fall as the estrogen–receptor complex apparently moves into the nucleus. After several

* From the Department of Physiology and Biophysics, University of Illinois, Urbana, Illinois 61801. *Received November 6, 1970.* Supported by National Institute of Health Grant HD 04889 and a U. S. Public Health Service predoctoral fellowship. An abstract of portions of this work has been published (Sarff and Gorski, 1969).

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hours the receptor numbers gradually rise. Jensen *et al.* (1969) reported that replenishment seen at 6 hr after estrogen can be prevented by cycloheximide; however, this would appear to be too short a time period to determine whether replenishment was or was not occurring. Studies reported below were undertaken to define more clearly how cytoplasmic estrogen receptor concentrations in the uterus are controlled with or without exposure to estrogen.

Methods

Preparation of Animals. Immature female rats, 22–25-days old (Holtzman or Sprague-Dawley), were used in the study. 17β-Estradiol (Mann Research), 0.1 μg of 0.5 ml of saline (0.154 M NaCl) and 0.2% ethanol, or vehicle alone was injected intraperitoneally. Where indicated, 0.1 μg of [6,7-^{3}H]17-β-estradiol (40–45 Ci/mmole, New England Nuclear) was used in place of the unlabeled 17β-estradiol. Cycloheximide (Sigma) (200 μg in 1.0 ml of saline/50 g of rat) was injected intraperitoneally either alone or at indicated times before or after 17β-estradiol. Actinomycin D (400 μg in 1.0 ml of saline/50 g of rat, gift of Merck) was injected intraperitoneally 60 min prior to or at indicated times after 17β-estradiol. Animals were maintained on food and water *ad lib.*

In Vitro Incubation of Whole Uteri. Whole uteri from untreated rats were incubated in Eagle's HeLa medium with glutamine added (Eagle, 1955) plus 10% hypophysectomized lamb serum and 200 U of penicillin G (Squibb) under an atmosphere of 95% O_2–5% CO_2 in a shaking water bath at 37°. When indicated, uteri were incubated with 1.4×10^{-3} M cylcoheximide or they were preincubated at 4° in Eagle's HeLa medium plus 1.0×10^{-8} M [^{3}H]17β-estradiol for 2 min, rinsed in fresh Eagle's HeLa medium, and placed in the 37° incubation medium.

Sucrose Density Gradient Analysis. Animals were decapitated; uteri were immediately removed, stripped of adhering fat, and placed in cold 0.04 M Tris-Cl–0.0015 M Na_2EDTA (pH 7.4) at 4°. All procedures were performed at 2–4° unless otherwise noted. Four or five uteri were pooled and homogenized for 20–30 sec in 0.2 ml of 0.04 M Tris-Cl–0.0015 M EDTA buffer per uterus using a ground-glass Kontes conical homogenizer. The homogenates were centrifuged at 800g for 10 min, and the supernatant fraction was further centrifuged at either 105,000g for 90 min or 270,000g for 30 min to obtain the soluble fraction, designated the cytosol. The cytosol was incubated with [^{3}H]17β-estradiol (9.2×10^{-9} M) for 15 min and 0.2 ml was layered onto a 3.7-ml 5–20% linear sucrose density gradient in 0.01 M Tris-Cl (pH 7.4) at 4°. The gradients were centrifuged in an International B-60 using an SB-405 rotor at 220,000g for 12 hr, fractionated, and counted as previously reported (Toft and Gorski, 1966).

Pellet Binding Assay. The cytosol was prepared as for sucrose density gradients except that uteri were rinsed and homogenized in 0.04 M Tris-Cl–0.1 M KCl–0.004 M $MgCl_2 \cdot 6H_2O$ (pH 7.2) at 25°. The cytosol was assayed for estrogen binding activity according to Clark and Gorski (1969), except that 150 mg of neutral alumina, 100–200 mesh (Bio-Rad), was used as the pellet in place of the ground glass.

Sephadex G-25 Column Assay. Sephadex G-25 (superfine, Pharmacia) columns were made in siliconized (Siliclad, Clay-Adams) disposable 6-in. Pasteur pipets. Cytosol was prepared as for sucrose density gradient analysis and was incubated 15 min with [^{3}H]17β-estradiol (1.8×10^{-7} M). A 0.05-ml aliquot of cytosol and [^{3}H]estradiol plus 0.01 ml of the marker dyes, Dextran Blue (Pharmacia) and chlorophenol red (Matheson Coleman & Bell) in 10% ficoll (Pharmacia), were layered onto the column. The column was eluted with 0.01 M Tris-Cl–0.02% NaN_3 (pH 7.4) at 22°. The void volume (0.5 ml, marked by Dextran Blue) was collected in scintillation vials and counted in a Packard Tri-Carb scintillation counter at 20% efficiency using 3 ml of ethanol and 10 ml of scintillation fluid (1 l. of toluene, 5 g of 2,5-diphenyloxazole, and 0.3 g of 1,4-[bis-2-(4-methyl-5-phenyloxazolyl)]benzene (Packard) per vial.

Protein and RNA Synthesis. L-[^{14}C]Leucine (1 μCi, 175 mCi/mmole, Schwarz) was injected intraperitoneally into rats 1 hr prior to sacrifice. After decapitation the uteri were removed. For *in vitro* determination of the rate of protein synthesis, 0.05 μCi of L-[^{14}C]Leucine was added to the medium 1 hr before the end of the designated 37° incubation period. Uteri from *in vivo* and *in vitro* incubations were rinsed in 10 ml of cold 0.04 M Tris-Cl–0.0015 M Na_2EDTA buffer and homogenized in 3 ml of cold 10% trichloroacetic acid. The homogenate was centrifuged at 800g for 10 min, and the pellet was washed three times in cold 5% trichloroacetic acid, twice in 95% ethanol, once in ethanol–chloroform (1:2, v/v), and twice in ether. The pellet was dried, weighed, and digested in 0.5 ml of hydroxide of Hyamine 10-X (Packard) at 37°. The scintillation fluid (10 ml), toluene–2,5-diphenyloxazole–1,4-[bis-2-(4-methyl-5-phenyloxazolyl)]benzene, was added to the solubilized pellet, and the radioactivity was determined by a Packard Tri-Carb liquid scintillation counter at 40% efficiency. The ^{14}C counts per minute per milligram of trichloroacetic acid precipitable protein was used as an indication of the rate of protein synthesis in the uterus.

RNA synthesis in the rat uterus was measured 1 hr after intraperitoneal injection of 1 μCi of [^{14}C]cytidine (Schwarz) into rats. The uteri were rinsed, and homogenized in 3 ml of cold 10% perchloric acid. The homogenate was centrifuged, and the 800g pellet was washed three times in 5 ml of 5% perchloric acid and treated the same as the trichloroacetic acid pellet above. ^{14}C counts per minute per milligram of perchloric acid precipitable material was used as an indication of the rate of nucleic acid synthesis.

[^{3}H]17β-Estradiol in Nuclear Fraction. After homogenization of uteri treated with [^{3}H]17β-estradiol, the 800g pellet was washed three times in 3 ml of 0.04 M Tris-Cl–0.0015 M Na_2EDTA buffer and extracted twice with 3 ml of ethanol and once with 3 ml of ether. The extracts were combined and evaporated in a scintillation vial; 10 ml of scintillation fluid (toluene–2,5-diphenyloxazole–1,4-[bis-2-(4-methyl-5-phenyloxazolyl)]benzene) was added, and the sample was counted in a scintillation counter. This fraction contains the [^{3}H]-estradiol which is bound in the nucleus (Shyamala and Gorski, 1969).

Results

In order to determine the effectiveness of cycloheximide as a protein synthesis inhibitor in the rat uterus over a prolonged time period, L-[^{14}C]leucine incorporation into trichloroacetic acid precipitable material was determined. Figure 1A shows that protein synthesis is essentially inhibited 100% for a 12-hr period when whole uteri from immature rats are incubated *in vitro* with 1.4×10^{-5} M cycloheximide. An increased rate of protein synthesis occurred in uteri incubated without cycloheximide. This increase has been noted previously (Mueller *et al.*, 1958) and cannot be attributed to bacterial contamination. It can be partially eliminated by adjusting the pH of the Eagle's HeLa medium with 95% O_2–5% CO_2, to pH 7.2 before adding the tissue and then incubating the uteri at

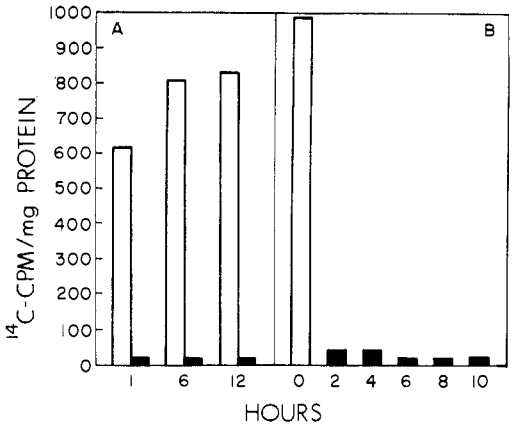

FIGURE 1: Effects of cycloheximide on uterine protein synthesis. (A) *In vitro* incorporation of 0.05 μCi of L-[^{14}C]leucine into trichloroacetic acid precipitable protein by immature rat uteri during the last hour of 37° incubation with cycloheximide (1.4×10^{-5} M, solid bars) and without cycloheximide (open bars). Results are from five pooled uteri per time period. (B) *In vivo* incorporation of 1 μCi of L-[^{14}C]-leucine into uterine trichloroacetic acid precipitable protein. [^{14}C]-Leucine was injected intraperitoneally into immature rats 1 hr before sacrifice. Cycloheximide (200 μg) was injected into each animal at 0 hr and at 5.5 hr. The results are from five pooled uteri per time period. The open bar represents saline-injected control; closed bars represent cycloheximide-treated groups.

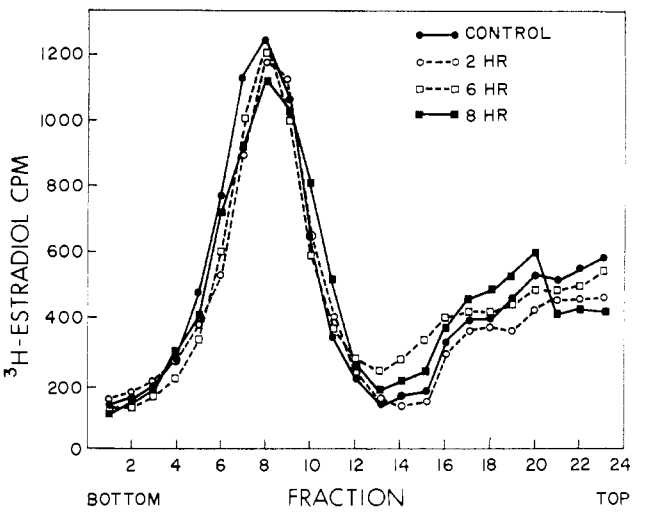

FIGURE 2: Sucrose density gradient pattern of uterine cytosol from immature rats treated 2, 6, or 8 hr with 200 μg of cycloheximide injected intraperitoneally. Five uteri were pooled per group. Gradients were centrifuged in an International B-60 centrifuge using an SB-405 rotor at 220,000g at 2° for 15 hr. (●—●) Control, (○- -○) 2 hr, (□- -□) 6 hr, (■—■) 8 hr.

37° under an atmosphere of air, rather than 95% O_2–5% CO_2, in a closed flask.

A single *in vivo* intraperitoneal injection of 200 μg of cycloheximide (1.4×10^{-3} mole) blocks protein synthesis almost 100% for up to 6 hr. After 6 hr, the rate of protein synthesis begins to increase, and by 12 hr after the injection of the inhibitor the rate is 60% that of the saline-injected control. Figure 1B shows that when cycloheximide is reinjected every 5–6 hr, protein synthesis remains almost 100% inhibited for 12 hr. Therefore, animals injected with cycloheximide every 5–6 hr were used in the *in vivo* experiments reported in this paper.

Results of *in vivo* studies using cycloheximide to inhibit protein synthesis were analyzed by sucrose density gradients. A representative experiment is shown in Figure 2. During 8 hr of protein synthesis inhibition, the profile of [^{3}H]17β-estradiol and cytosol on a sucrose density gradient does not change, and the estradiol sediments as an 8S peak in all cases. This constancy in the appearance of the 8S peak suggests that the inhibitor alone does not adversely affect the binding of 17β-estradiol to the receptor, nor does it appear to change the properties of the receptor as seen on a sucrose density gradient. The binding ability of the cytoplasm dropped only 4–5% during the 8-hr period of protein synthesis inhibition. Similar results were obtained using the glass pellet binding assay to measure cytoplasmic binding capability in uteri of rats treated with cycloheximide. Longer term *in vivo* studies were not performed because of the toxicity of the antibiotic drug to the animals.

Incubation of uteri with or without cycloheximide for up to 20 hr had no effect on the sedimentation velocity of the 8S peak. Using Sephadex G-25 columns to separate bound from free [^{3}H]17β-estradiol, the amount of bound estradiol in the cytosol decreases approximately 10% during the 20-hr incubation period with cycloheximide present as compared to cytosol from uteri incubated without the inhibitor.

Figure 3 shows the mean plus and/or minus standard error of ten experiments done either *in vivo* or *in vitro* and assayed by sucrose density gradients, Sephadex columns, or pellet binding. Data are presented as per cent of the control; *in vivo* controls were saline injected and *in vitro* controls were incubated without cycloheximide for the same time as the experimental groups. In three experiments two assay methods were used; the average value was determined for the binding from the two methods, and this average was then used in calculating the values in Figure 3. The means at 8, 10, and 12 hr are significantly less than the control when $p < 0.1$. Assuming that the degradation of the receptor follows first-order kinetics, one can calculate from these data the half-life of the receptor to be 5 days or longer. Because the period of blocked protein synthesis is relatively short and the decrease in binding small, this estimate is of limited precision, but it does indicate that the receptor is a relatively long-lived molecule.

Figure 4 shows the time course of 17β-estradiol binding capacity in uterine cytosol after intraperitoneal injection of 0.1 μg of 17β-estradiol at 0 hr. Animals were killed at the designated times after 17β-estradiol injection and assays for [^{3}H]17β-estradiol binding capacity were performed using

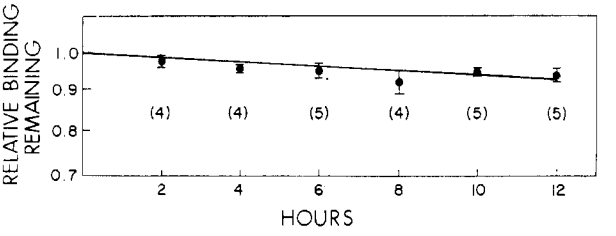

FIGURE 3: Relative [^{3}H]17β-estradiol binding capability in uterine cytosol of immature rats during exposure to cycloheximide. Data from ten experiments are expressed as the mean of the per cent of control plus and/or minus standard error. *In vivo* controls were saline injected, and *in vitro* controls were incubated without cycloheximide for the same period as the experimental groups. Assays for [^{3}H]17β-estradiol binding were done either by sucrose density gradients, the glass pellet binding assay, or Sephadex G-100 columns. 8, 10, and 12 hr means are significantly less than control 0.1 level. ( ) indicate number of experiments used to determine mean.

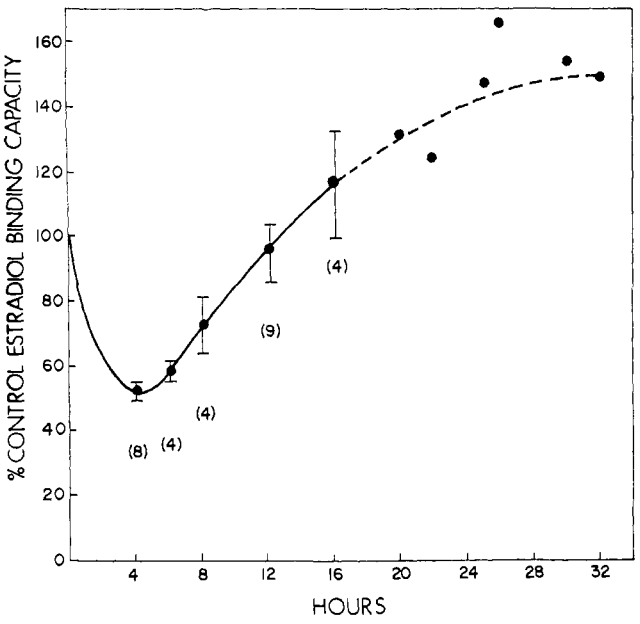

FIGURE 4: Time course of estrogen binding capacity in the cytoplasm after estrogen injection. 17β-Estradiol (0.1 μg) was injected intraperitoneally into rats at 0 hr. Assays for estradiol binding capacity used sucrose density gradients, pellet binding, or Sephadex columns as described in Methods. Results are expressed as the mean plus and/or minus standard error of per cent of saline-injected control. ( ) indicate the number of experiments used in calculating the mean plus and/or minus standard error. Points shown beyond 16 hr are single determinations.

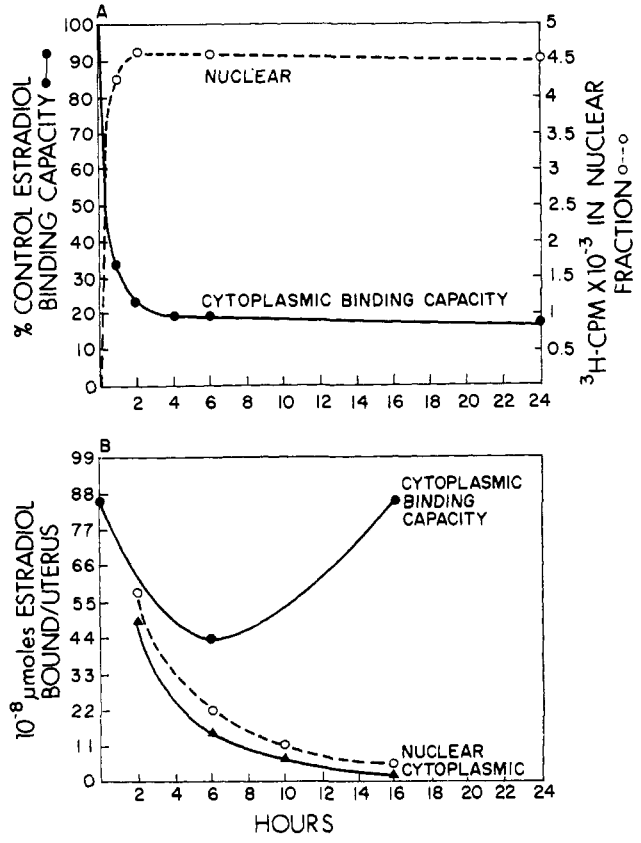

FIGURE 5: Time course of subcellular distribution in uterus of estrogen after *in vitro* or *in vivo* administration of the hormones. (A) Total cytoplasmic and nuclear binding after an *in vitro* pulse of [^{3}H]17β-estradiol. Uteri were pulsed with 1.0 × 10^{-8} M [^{3}H]17β-estradiol at 4° for 2 min followed by 37° incubation in estrogen-free Eagle's HeLa medium. Binding was measured by Sephadex G-25 columns as described in Methods. Cytoplasmic binding capacity determined in the cytosol after the pulse of estradiol was compared to the binding capacity of cytosol with no previous estradiol exposure (●—●). Nuclear ^{3}H binding remaining after the estradiol pulse and subsequent incubation was determined as described in the text and is expressed as ^{3}H counts per minute per uterus (O– –O). Each point is the mean of three samples of two uteri per sample. (B) [^{3}H]17β-Estradiol remaining in the 800*g* pellet (O– –O) and supernatant (▲—▲) after intraperitoneal injection of 0.1 μg of [^{3}H]17β-estradiol. Total cytosol binding was measured using the pellet assay as described in Methods (●—●). Results are expressed as 10^{-8} μmole of estradiol/uterus. Each point represents the mean of four individually assayed uteri.

sucrose density gradients, pellet binding, or Sephadex columns as described in Methods. Data from 11 experiments were used to compute the mean plus and/or minus standard error of the per cent of saline-injected control. The values found for 4, 6, and 8 hr are not significantly different from one another at the 0.01 level using Student's t test. The 12-hr binding, however, is significantly different from the 4-, 6-, and 8-hr points at the 0.01 level. By 16 hr after estrogen injection, the amount of cytoplasmic receptor capable of binding [^{3}H]-17β-estradiol has returned to control values and, subsequently, an overshoot occurs. A similar picture is seen when the data are expressed as [^{3}H]17β-estradiol bound/100 μg of DNA.

The effect of *in vitro* estradiol on the receptor concentration was also studied. Figure 5A shows the estradiol binding capacity of uterine cytosol after estradiol as compared to the binding capacity of uteri not exposed to estrogen. The ratio drops rapidly during the first few hours of *in vitro* incubation, levels off, and remains quite constant up to 24 hr when the experiment was terminated. When an aliquot of the cytosol was counted 1 hr after the pulse of [^{3}H]estradiol, only 10% of the original counts in the cytosol remained. By 6 hr after the pulse, the cytosol had no detectable radioactivity. [^{3}H]Estradiol in the nuclear fraction, after *in vitro* incubation, was determined and is also shown in Figure 5A. The nuclear binding reaches a peak at 2 hr and remains at a high level for the remainder of the 24-hr period studied.

After an *in vivo* intraperitoneal injection of 0.1 μg of [^{3}H]-17β-estradiol, there is a rapid fall in the radioactivity, bound or free, found in the 800*g* supernatant (cytoplasmic) and nuclear fractions of the uterus as shown in Figure 5B. Each point represents the mean of four individual uteri. Also shown is the total binding capacity of the cytosol after the injection of [^{3}H]17β-estradiol as determined by the pellet binding assay which employs an excess of [^{3}H]17β-estradiol to ensure [^{3}H]-estradiol binding to all the "receptors" in the cytosol. Between 6 and 16 hr after estrogen injection, the binding capacity in the cytosol increases by about 34 × 10^{-8} μmole of estradiol, while during the same time the residual nuclear binding decreases by only 16 × 10^{-8} μmole of estradiol. Therefore, the increase in cytoplasmic binding ability during this period cannot be attributed solely to the loss of estradiol binding in the nucleus. However, from Figure 5A it would appear that perhaps a decrease in nuclear binding is in some way involved in the return of binding in the cytoplasm, but that an essential component in the system is absent under the *in vitro* conditions used for this study.

In order to examine the role of protein synthesis in the replenishment of the cytoplasmic estrogen receptor, the protein synthesis inhibitor cycloheximide was injected 10 min before or 2, 4, or 6 hr after the injection of 0.1 μg of 17β-estradiol.

TABLE 1: [^{3}H]17β-Estradiol Binding by Uterine Cytosol after 0.1 μg of 17β-Estradiol Was Injected with or without Cycloheximide *in Vivo* for the Designated Time Periods (*e.g.*, 2–12 hr; Cycloheximide Injected 2 hr after Estrogen and 10 hr before Sacrifice).[a]

		12-hr Estradiol Cycloheximide Exposure (hr)				
Control	4-hr Estradiol	None	0–12	2–12	4–12	6–12
100	50	98	48		62	
100	56	90	49	58	70	
100	45	104	45		60	
100	43	98	42	40	68	
100			35	40		76

[a] Results are expressed as the per cent of saline-injected control and are the mean of three or four samples. [^{3}H]17β-Estradiol binding was measured using pellet binding or Sephadex G-25 columns or both, as described in Methods.

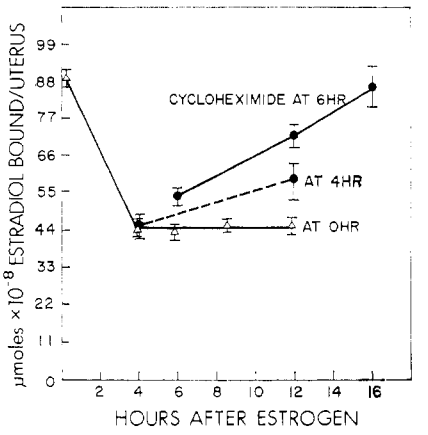

FIGURE 6: Binding to cytosol after injection of 0.1 μg of [^{3}H]17β-estradiol at 0 hr and 200 μg of cycloheximide at 10 min before estrogen (△—△), 4 hr after estrogen (●- -●), and 6 hr after estrogen (●—●). Each point represents the mean plus and/or minus standard error of four experimental means. Each experimental mean was calculated from three or four uterine pellet binding assays as described in Methods.

Table I presents data from five such experiments. The results are expressed as per cent of saline-injected control measured by the pellet binding assay or Sephadex G-25 columns, or both. The data indicate that when cycloheximide is injected shortly before or 2 hr after estradiol, return of the estrogen binding receptor is inhibited, but that if cycloheximide is injected 4 or 6 hr after estradiol, the replenishment of estrogen binding capacity occurs in the presence of the inhibitor. Figure 6 shows the rate at which binding returns to the uterine cytosol, as determined by the pellet binding assay, when cycloheximide is injected 0, 4, or 6 hr after estradiol. The rate of increase ot the controls is $4.1 \pm 0.5 \times 10^{-8}$ μmole of estrogen/hr; for those injected with cycloheximide 4 hr after estrogen, it is $1.8 \pm 0.5 \times 10^{-8}$ μmole of estrogen/hr; and for those injected with cycloheximide 6 hr after estrogen, the rate of increase is $3.4 \pm 0.4 \times 10^{-8}$ μmole of estrogen/hr. The difference is not statistically significant between controls and uteri injected with inhibitor 6 hr after estrogen, nor between those injected with inhibitor 4 and 6 hr after estrogen; but there is a statistical difference between the rate of increase of the controls and those injected with cycloheximide 2 or 4 hr after estrogen at $p < 0.01$. This indicates that between 2 and 6 hr after estrogen injection (during the time when estrogen binding capacity in the cytoplasm reaches a low point), an event takes place which is cycloheximide sensitive, and which is essential for the subsequent replenishment of receptor in the cytoplasm. However, the replenishment process, occurring between 6 and 16 hr after estrogen, does not involve protein synthesis.

Sucrose density gradient analysis was used to investigate the "state" of the receptor made in the presence of cycloheximide. Cytosol was prepared from animals treated with estrogen alone or with estrogen plus cycloheximide given 2 and 6 hr after estrogen. Figure 7 shows that [^{3}H]estradiol binding in the 8S peak decreases to a low level at about 6 hr after estrogen and then rises until, at 16 hr after estrogen, the binding in the 8S peak has returned to control values. When cycloheximide was injected 2 hr after estrogen, the binding in the 8S peak remained at the low level for the 8-hr period studied. However, when cycloheximide was injected 6 hr after estrogen and the binding was assayed 10 hr later, binding in the 8S peak had increased from the low level found at 6 hr after estrogen. Under the assay conditions, the 8S estrogen receptor decreases after estrogen treatment and subsequently increases again, still as an 8S receptor, even when protein synthesis is inhibited during this period of replenishment.

An increase in estrogen associated with the 4S region of the gradient is likely due to the hyperemia of the uterus after cyclo-

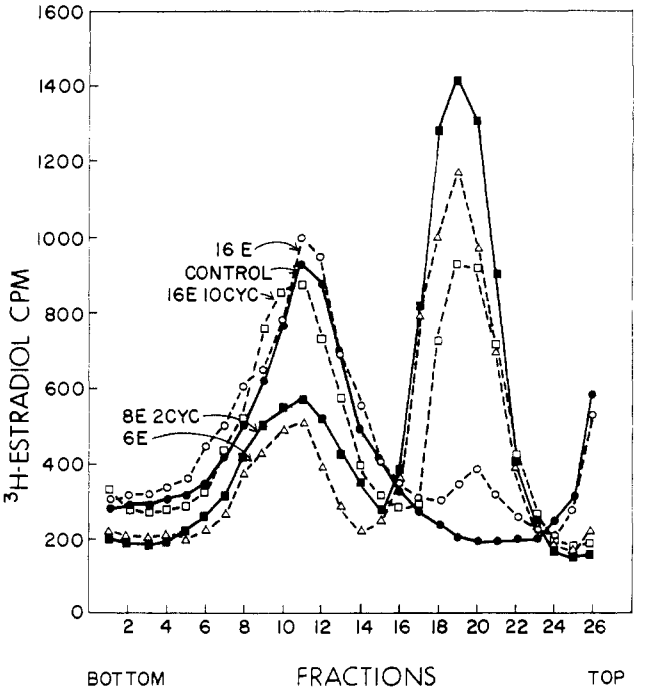

FIGURE 7: [^{3}H]17β-Estradiol binding to cytosol as assayed by sucrose density gradients. Animals were injected with 0.1 μg of estradiol for 6 hr (△- -△) or 16 hr (○- -○), and with 280 μg of cycloheximide 6 hr after estrogen—assayed 8 hr (■- -■) and 16 hr (□- -□) after estrogen. Control (●—●) was saline injected at 0 and 6 hr. Sucrose density gradients were prepared as described in Methods and centrifuged in an International B-60 using an SB-405 rotor at 220,000g at 2° for 12 hr.

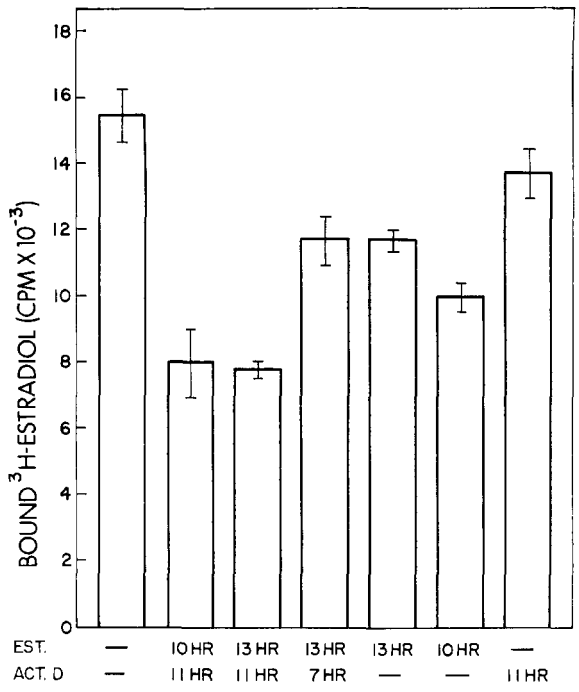

FIGURE 8: [^{3}H]17β-Estradiol binding to uterine cytosol assayed using pellet binding as described in Methods. Rats were injected with 400 μg of actinomycin D at 1 hr prior to or 2 or 6 hr after a 0.1 μg of actinomycin D at 1 hr prior to or 2 or 6 hr after a 0.1 μg of injection of 17β-estradiol. Results are expressed as the mean plus and/or minus standard error of four samples per group.

heximide treatment. This 4S estrogen was not associated with high-affinity binding as measured by the pellet binding assay.

To investigate the effect RNA synthesis has on the receptor, actinomycin D was used to inhibit RNA synthesis. Actinomycin D (400 μg) inhibited incorporation of [^{14}C]cytidine into total uterine RNA by 93% for up to 10 hr. Actinomycin D by itself caused a slight but statistically insignificant decrease in total binding of controls (Figure 8). When actinomycin D was injected 1 hr prior to or 2 hr after estrogen, the normal drop in estrogen binding occurred, but there was no subsequent increase. In contrast, when actinomycin D was injected 6 hr after estrogen, a normal return of estrogen binding occurred. The data suggest that an actinomycin D sensitive step (presumably the synthesis of RNA) occurs between 2 and 6 hr after estrogen injection (during the low point in cytoplasmic estrogen binding ability), which is necessary for the subsequent increase in receptor binding ability in the cytoplasm. However, RNA synthesis is not necessary during the period of most rapid increase in receptor binding capacity.

Discussion

The regulation of the estrogen receptor concentration in the rat uterus has turned out to be a rather complex process. The data show that during 8–12 hr of continued protein synthesis inhibition, the concentration of the estrogen receptor, as reflected by its ability to bind [^{3}H]estradiol, changes only approximately 6% from control levels whether the inhibitor is present *in vivo* or *in vitro*. If the assumptions are made that cycloheximide is affecting the receptor number and activity only by its inhibition of receptor synthesis, the rate of degradation is a reflection of the turnover rate of the receptor molecule, and the half-life of the receptor, using $t_{1/2} = \ln 2/k$, is approximately 5 days. Although this is an approximate value, the half-life is much longer for the estrogen receptor than the half-life of 20–22 hr determined for the total uterine cytosol proteins (Gorski and Notides, 1969).

If evenly distributed throughout the tissue, the number of estrogen binding sites can be calculated to be ~16,000 molecules/cell under basal conditions both in 20- to 30-day-old immature rats (Clark and Gorski, 1970) and in ovariectomized rats (Notides, 1970). Thus, under basal conditions where synthesis equals degradation, the synthesis of estrogen binding sites is calculated to be equal to 80 binding sites/hr per cell.

Studies on the ontogeny of the estrogen receptor in the rat uterus (Clark and Gorski, 1970) have shown a marked increase in the concentration of estrogen binding sites between days 1 and 10 of life, which could be accounted for by a rate of synthesis of approximately 151 sites/hr per cell using the formula $S = k(P' - Pe^{-kt})/(1 - e^{-kt})$ (Russell and Snyder, 1969). Furthermore, Berlin and Schimke (1965) have calculated that the half-life of a protein is equal to half the time it takes to reach equilibrium after the rate of synthesis is changed. If one assumes that change in rate of synthesis starts about the time of birth, or perhaps 1 or 2 days earlier, one can conclude that the half life of the binding protein is approximately 5 days. These estimates of rates of synthesis and half-life are surprisingly close to the estimates derived in this study.

The possibility cannot be ruled out, however, that the receptor is degraded by an enzyme which has a short half-life, and that the synthesis of this enzyme is inhibited by cycloheximide in addition to synthesis of the receptor itself being inhibited. This explanation indeed seems to be true in a few specific cases, the best documented being tyrosine transaminase (Kenney, 1967; Schimke, 1967; Levitan and Webb, 1970), but it does not seem to be applicable to proteins in general (Russell and Snyder, 1969; Feldman and Yagil, 1969).

In contrast to the rather stable levels of estrogen receptor found in the cytoplasm during the absence of estrogen, after estrogen stimulation the receptor concentration in the cytoplasm of rat uterine cells changes significantly and quite rapidly. There appear to be three stages in the cytoplasmic concentrations of estrogen binding protein that follows estrogen administration: (1) depletion, (2) macromolecular synthesis, and (3) replenishment.

Previous work has shown that the cytoplasmic receptor binds estrogen and then moves into the nucleus by an unknown temperature-dependent process, and this could explain the depletion process. Under *in vitro* conditions with saturating levels of estrogen, this process results in the loss of 80% of the binding protein from the cytoplasm within 1 hr. Under *in vivo* conditions where saturation is difficult to achieve and where estrogen is being made available over a period of time, this process takes 4–6 hr as reported in this study.

The second stage is most tenuous because it is based on a simplistic interpretation of the action of protein and RNA synthesis inhibitors. The events occurring during this period must be able to explain the long replenishment process, which can occur in the face of almost complete inhibition of RNA or protein synthesis.

Synthesis of the receptor in an inactive form could be occurring during this period. The data indicate that essentially all the necessary macromolecules are synthesized between 2 and 6 hr after estrogen. This would necessitate that the rate of synthesis be ~2000 binding sites/cell per hr or 20 times the basal synthetic rate as estimated by the slow turnover of the estrogen receptor under equilibrium conditions. The time course of receptor replenishment indicates a slow activation of the receptor after synthesis has been completed.

Another explanation of the data is that an enzyme is synthesized and it then produces a vital component of the receptor, perhaps a ligand which can activate an inactive receptor. No evidence exists for a ligand being bound to the activated receptor; however, our knowledge of its chemistry either with or without bound estrogen is too meager to eliminate this possibility.

The accumulating evidence that the receptor consists of subunits (Erdos, 1968; Jensen *et al.*, 1969) is compatible with the idea of a conformational change being induced to yield an active receptor from an inactive form.

A reciprocal relationship between replenishment and the loss of estrogen from the nucleus is intriguing but far from clear. Such a relationship would involve a lag of approximately 2 hr and is supported principally by the observation that, during *in vitro* incubations following estrogen administration, neither loss of estrogen from the nucleus nor replenishment occurs.

The relatively long half-life of the estrogen receptor in the absence of estrogen indicates a slow rate of degradation. Thus, a reasonable change in the rate of degradation would still not not be expected to bring the rapid changes in receptor concentrations seen in this study. The degradation rate of the receptor could, however, be markedly changed when the receptor is combined with estrogen or moves into the nucleus.

If the estrogen binding protein of the uterus is truly a receptor molecule with its accompanying physiological importance, then the regulation of the receptor itself is bound to be of interest. The studies reported here suggest that the regulation of the receptor will be as complicated to decipher as the mechanism of action of the receptor.

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Transfer Ribonucleic Acid Methylase Activity in the Developing Pig Brain*

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ABSTRACT: tRNA methylase activity in whole brain preparations from adult pigs was found to be very low (~10 $\mu\mu$moles/30 min per mg of protein), whereas examination of five separate regions, frontal cortex, cerebellum, pons medulla, midbrain, and corpus callosum, revealed some regions to be three to four times more active than that reported for the whole brain preparation.

Methylase activity was found to be high in fetal brain and to decrease in older animals after birth. A tRNA methylase inhibitor was also found in fed newborn pigs as early as 12 hr after birth and in adult animals. It was absent from fetal tissue and its synthesis or activation could be delayed for up to 48 hr in newborn pigs fasted immediately after birth.

Transfer RNAs have molecular weights between 25,000 and 30,000 and contain in addition to the normal nucleotides over 30 minor bases. Some of these are derived by the methylation of the 4 main bases at the polynucleotide level by the tRNA methylase enzymes. These enzymes are base specific, site specific, and evidence is accumulating that indicates that they are sequence specific as well (Srinivasan and Borek, 1963; Baguley and Staehelin, 1969; Kuchino and Nishimura, 1970).

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