Replenishment of uterine estrogen receptor was examined in immature rats following injection of 16α-estradiol. 16α-Estradiol is a "short-acting" estrogen that, after a single injection, stimulates early estrogenic responses (water imbibition, induced protein synthesis, etc.), but not long term responses (DNA synthesis). Replenishment after 16α-estradiol injection was complete within 4 h. Furthermore, disappearance of receptor from the nucleus closely corresponded to a reappearance of receptor in the cytoplasm. In contrast to this, receptor replenishment following injection of 1 µg of either diethylstilbestrol or 17β-estradiol was very slow and lagged behind the disappearance of nuclear receptor, leading to an apparent decrease in total receptor content. Half-lives for the clearance of nuclear estrogen-receptor complexes were estimated to be 30 min for 16α-estradiol and 2 h for 17β-estradiol, respectively. Inhibition of protein synthesis by cycloheximide did not inhibit replenishment after 16α-estradiol injection. Studies on replenishment after 17β-estradiol injection in the presence of cycloheximide could not be interpreted due to a decrease in total receptor content caused by long term cycloheximide treatment. Multiple injections of 16α-estradiol did not lead to a lag in replenishment time or a decrease in total receptor content. This represents a case in which estrogen receptor replenishment appears to be due entirely to receptor recycling.

According to current models of estrogen action, estrogen binds to a cytoplasmic receptor causing receptor "transformation" which results in the translocation of the estrogen-receptor complex into the nucleus (1–3). This initial depletion of cytoplasmic receptors is followed by a gradual replenishment of receptors in the cytoplasm. Replenishment of receptors is important in rendering tissues responsive to further estrogen stimulation (4).

The mechanism of replenishment has been a controversial topic since 1969 when Jensen et al., demonstrated that cycloheximide could inhibit receptor replenishment that occurred after 17β-estradiol administration (5). Subsequent studies on receptor replenishment following 17β-estradiol injection have all shown at least partial inhibition of replenishment by protein-synthesis inhibitors (6–8). The problem with studying receptor replenishment following 17β-estradiol administration is 2-fold. First, replenishment after exposure to 17β-estradiol is very slow, requiring 11 to 16 h for recovery of control receptor levels (4, 10); therefore, in order to study inhibition of replenishment, inhibitors must be present for long periods of time. Animals treated with cycloheximide are under severe stress and often die after 12 h or more of exposure. Interestingly, Sarff and Gorski (10) observed that administration of cycloheximide prior to 17β-estradiol treatment completely blocked receptor replenishment that occurred 6 to 12 h later. However, administration of cycloheximide 6 h after the administration of 17β-estradiol (when cytosol receptor content was still very low) did not affect subsequent replenishment. Although these results could indicate that replenishment occurs in two phases: an early, protein synthesis-dependent phase, followed by a later, protein synthesis-independent phase, they could also be due to general toxic effects of cycloheximide at later times after administration. Therefore, results obtained in animals which have been treated with inhibitors for long periods of time must be interpreted with caution. The second problem encountered when using 17β-estradiol to study replenishment is that one response to 17β-estradiol is an increase in total estrogen receptor levels. This increase apparently is due to the synthesis of new estrogen receptors and is connected with the cellular hypertrophy and hyperplasia caused by 17β-estradiol (11). Thus, separating what is happening to "old" receptors from the synthesis of "new" receptors is difficult.

It has been recently shown that replenishment after some short-acting estrogens is very rapid. Martucci and Fishman have shown that replenishment after a single injection of 2-hydroxyestradiol is complete within 3 h (12). In this laboratory, Stack and Gorski (8) have found that replenishment after injection of 16α-estradiol is complete within 4 h. Furthermore, loss of receptor from the nucleus closely corresponds to reappearance of receptor in the cytoplasm. This suggests that replenishment of the estrogen receptor following 16α-estradiol injection is due entirely to receptor recycling. Jungblut (8) has noted a similar pattern for the estrone-receptor complex. In the studies reported here, we have investigated the effect of the protein synthesis inhibitor cycloheximide on replenishment following injection of 16α-estradiol. Since replenishment occurs at such a rapid rate, it is possible to perform these experiments under conditions when the toxic effects of cycloheximide are not apparent. We found that replenishment following 16α-estradiol is not dependent on protein synthesis and therefore may be due entirely to receptor recycling.

**Experimental Procedures**

Animals and Treatments—Immature female rats (21 to 22 days old) were obtained from King Co. (Madison, WI). The rats were maintained on a 12 h light, 12 h dark cycle and were given food and water ad libitum. Animals were treated with estrogen or estrogen and cycloheximide as described below.

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1 G. Stack, and J. Gorski, manuscript in preparation.
water ad libitum. All injections were given intraperitoneally as follows: 16α-estradiol (2.5 pg), estradiol (2.5 pg), diethylstilbestrol (1 μg), and 17β-estradiol (1 μg) in 0.25 ml of 0.9% NaCl plus 10% ethanol; cycloheximide (200 μg) in 1 ml of 0.9% NaCl; and [1H]leucine (4 μCi) in 0.25 ml of 0.9% NaCl. Controls were injected with vehicles alone.

Chemicals—17β-[6,7-3H]Estradiol (40 Ci/mmol), 17β-[2,4,6,7-3H]estradiol (101 Ci/mmol), and [1H]leucine (55 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA). Stock solutions of the above radioisotopes were made in absolute ethanol and stored at −20°C. Diethylstilbestrol, dithiothreitol, cycloheximide, Tween-80, 17β-estradiol, and estriol were purchased from Sigma Chemical Co. (St. Louis, MO). 16α-Estradiol was a gift from Park Davis, Co. (Ann Arbor, MI). Hydroxyapatite was obtained from Bio-Rad Laboratories and prepared by washing with 0.05 M Tris-HCl (pH 7.3) at 25°C until pH of the wash was equal to 7.2.

The buffers used were TED (10 mM Tris-HCl, 1.5 mM EDTA, 0.5 mM dithiothreitol), TEDT (TED plus 0.2% Tween-80), and T (50 mM Tris-HCl). Each buffer was adjusted to pH 7.3 at 25°C. Regular scintillation fluid contained 15.0 g of POP (2,5-diphenyloxazole) and 0.9 g of POPOP (1,4-bis[2-(5-phenyloxazolyl)]benzene) in 3 liters of toluene. Ten% BBS-3 scintillation mixture contained BBS-3 (Beckman) 10% (v/v) in regular scintillation fluid.

Preparation of Tissue Fractions and Receptor Assays—Animals were decapitated, uteri removed, stripped of mesentery, and placed in ice-cold TED buffer. All subsequent steps were performed at 4°C. Each uterus was homogenized in TED buffer (one uterus per ml) with a Kontes Dual1 type tissue grinder. Two aliquots (0.4 ml each) were taken from each homogenate (one for determination of total binding, one for determination of nonspecific binding) and centrifuged at 800 g for 20 min at 25°C. The crude nuclear pellets were treated as described below. For cytosol assays, 0.2 ml of each supernatant was added to 0.2 ml of 30 nM [3H]estradiol with or without a 100-fold excess of diethylstilbestrol. These samples were incubated for 18 to 24 h at 25°C to label both filled and unfilled estrogen receptor (13). At the end of the incubation, samples were placed on ice for 15 min and 0.2 ml of hydroxylapatite slurry (0.6 ml of packed hydroxylapatite/ml) was added. Tubes were processed as described by Williams and Gorski (14) except that hydroxylapatite pellets were extracted overnight with 1 ml of absolute ethanol, and 0.5 ml was counted in 3.5 ml of BBS-3 scintillation fluid at an efficiency of 30%.

The pellets obtained after centrifugation (above) were washed three times with 2 ml of TED buffer. Four-tenths ml of TED buffer containing 15 nM [3H]estradiol with or without 1.5 μM diethylstilbestrol was then added to each tube. Nuclear exchange assays were performed at 37°C for 30 min as described by Anderson et al. (15). Samples were washed on ice, washed four times with TEDT, and the final pellet was extracted with 1 ml of absolute ethanol overnight. Samples (0.5 ml) were counted in 3.5 ml of BBS-3 at an efficiency of 30%. Specific estrogen binding was calculated by subtracting nonspecific binding ([3H]estradiol plus 100-fold excess diethylstilbestrol) from total binding ([3H]estradiol only). DNA was determined by the method of Burton (16) using calf thymus DNA as a standard.

Protein Synthesis—Various times after treatment with cycloheximide, rats were injected with [1H]leucine. One h after leucine injection, animals were decapitated, uteri excised and rinsed in ice-cold 0.9% NaCl; all subsequent steps were at 4°C. Each uterus was homogenized in 1 ml of 0.5 M perchloric acid. Two aliquots of 0.4 ml were taken from each homogenate, one for DNA determination and one for determination of [1H]leucine incorporation. Samples were put on ice for 20 min and then washed three times with 2.0 ml of 0.3 M perchloric acid, centrifuging at 800 × g for 10 min between washes. One ml of 2N NaOH (Amersham) was added to each leucine incorporation tube; pellets were allowed to digest to completion and 9 ml of regular scintillation fluid was added. Samples were counted at an efficiency of 30%.

RESULTS

Receptor Replenishment after Administration of 17β-Estradiol, Diethylstilbestrol, Estradiol, and 16α-Estradiol—The nuclear and cytoplasmic receptor levels obtained after injection of various estrogenic compounds is shown in Fig 1. Injection of 17β-estradiol or diethylstilbestrol is followed by a rapid depletion of receptors from the cytoplasm with a concomitant increase in nuclear receptor (Fig. 1A). After injection, nuclear receptor levels have dropped to 50% of their value at 30 min, whereas cytoplasmic receptor levels remain depressed. The amount of nuclear receptor continues to decrease, so that by 4 h there is only 30 to 40% of the amount at 30 min. Cytoplasmic receptor levels remain depressed and thus total receptor (nuclear plus cytoplasmic) decreases in this time period. This decrease in receptor can be as much as 60%. This observation has been reported by other investigators (9, 19), and may be due to receptor degradation, inactivation, or inability of the exchange assay to measure all available receptors.

16α-Estradiol gave an entirely different result. As with 17β-estradiol and diethylstilbestrol, nuclear receptor levels rise rapidly in response to injection, but by 2 h are back down to control. Cytoplasmic receptors are rapidly replenished, such that by 2 h they are almost back to control. Receptor replenishment after injection of estradiol occurs at an intermediate rate.

Replenishment during Inhibition of Protein Synthesis—Using a dose of cycloheximide previously reported to be effective (7, 10), protein synthesis was inhibited greater than 85% for 4 h after injection (Fig. 2). The effect of cycloheximide on protein synthesis was immediate; injection of cycloheximide 10 min before injection of [1H]leucine inhibited protein synthesis 96%.

Fig. 3A shows the effect of cycloheximide on cytoplasmic receptor. Four h of treatment with cycloheximide did not decrease in nuclear receptor. Two h after injection, nuclear receptor levels have dropped to 50% of their value at 30 min, whereas cytoplasmic receptor levels remain depressed.
Estrogen Receptor Replenishment

TIME AFTER CYCLOHEXIMIDE TREATMENT

FIG. 2. Inhibition of protein synthesis by cycloheximide. Animals (5 rats/group) were injected with [3H]leucine at various times after cycloheximide or saline (control) injection and treated as described under "Experimental Procedures." Values are expressed as mean counts per min incorporated per pg of DNA ± standard error.

Fig. 3. Effect of cycloheximide on uterine receptor content. Rats were killed 1 and 4 h after injection with cycloheximide (200 μg/rat) and uterine receptor levels were measured as described. A, cytosol receptors; B, nuclear receptors. E, estradiol.

Extremely variable. Ciidlowski and Muldoon (17) have reported a similar decrease in the amount of uterine receptor after prolonged treatment of rats with cycloheximide, and have postulated that it is due to the general toxicity of the drug. It may be of some interest to note that cycloheximide causes a decrease in the already small amount of receptor which is present in the nucleus before the administration of estrogen (Fig. 3B). The significance of this is unclear.

Having established that protein synthesis is effectively inhibited, and that the amount of receptor is stable during the first 4 h of cycloheximide treatment, we examined replenishment after treatment with 16α-estradiol in the presence of cycloheximide. Fig. 4 illustrates that cycloheximide has little effect on receptor translocation into the nucleus (Fig. 4A) and little effect on cytoplasmic receptor replenishment (Fig. 4B). Therefore, replenishment after injection of 16α-estradiol occurs independently of protein synthesis.

Experiments designed to compare directly cycloheximide's effect on replenishment after 17β-estradiol injection versus its effect on replenishment following 16α-estradiol injection gave an interesting result (Fig. 5). Although, as seen above, cycloheximide had no effect on 4 h replenishment of 16α-estradiol, the level of cytosolic receptors dropped dramatically after 10 h of treatment. This is probably due to a decrease in the total amount of receptors caused by cycloheximide. While this may be due in part to inhibition of protein synthesis, the general toxicity of the compound apparent in the appearance of the animals and their gastrointestinal tracts, and even death in some cases by 10 h, makes it impossible to judge cause and effect relationships. Examining cycloheximide's effect on replenishment after 17β-estradiol thus gave meaningless results. This experiment clearly shows the dangers involved in long term inhibitor studies.

Effect of Multiple Injections of 16α-Estradiol on Replenishment—16α-Estradiol-receptor complexes are cleared from the nucleus with an estimated half-life of 30 min, while 17β-estradiol-receptor complexes have a half-life in the nucleus of around 2 h. To investigate the question of whether the delay
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in replenishment after 17β-estradiol treatment was a function of the higher levels of receptor-estrogen complex maintained in the nucleus by this hormone, rats were injected with 2.5 μg of 16α-estradiol at 0, 1, and 2 h. This injection scheme maintains nuclear receptor levels equal to that obtained from one injection of 17β-estradiol, based on the estimated half-lives of the nuclear receptor complexes (given above). As can be seen in Fig. 6A, replenishment 4 h after the last of the three injections of 16α-estradiol (6 h after the initial injection) is just as rapid as if only one injection were given. As shown in Figs. 6, A and B, low cytosol receptor levels at 0.5 h after the third injection (2.5 h after the initial injection) were matched by high nuclear levels. The sum of nuclear and cytosol receptors did not decrease from control by more than 20% (Fig. 6C). This is in marked contrast with what has been seen for 17β-estradiol where almost all receptors that have been translocated are unaccounted for or lost 4 to 6 h after injection.

DISCUSSION

The results that have been presented above indicate that replenishment after administration of 16α-estradiol is not dependent on protein synthesis and therefore may occur totally through receptor recycling. Estrogen receptor replenishment after 16α-estradiol treatment is very rapid and would require a very active protein synthesis to account for the observed accumulation of receptor protein in 2 h. Cycloheximide, at a dose that effectively blocked protein synthesis, had no effect on the time course or magnitude of replenishment which supports the concept that recycling is the principal factor in replenishment. It should be noted that the cycloheximide inhibition experiments assume that all protein synthesis, including receptor synthesis is blocked equally. At the time of replenishment, induced protein synthesis, etc.) but not long term responses. As shown by Stack and Gorski, multiple injections of 16α-estradiol, if properly spaced, do stimulate DNA synthesis equivalent to that observed after 17β-estradiol injection. However, the experiments reported here indicate that very little loss of receptor occurs even after multiple injections of 16α-estradiol. Thus, loss of total receptor may not be
important for estrogen action in the rat uterus. This has been noted by other investigators (9).

Clearly estrogen receptor replenishment can be accounted for fully through recycling, and receptor "processing" is not mandatory for growth of the uterus. However, the paradoxical loss of receptor and cycloheximide-sensitive replenishment after 17β-estradiol administration require further study.

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