*THE ROLE OF RNA SYNTHESIS IN EARLY ESTROGEN ACTION**

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Communicated by Henry Lardy, June 10, 1963

Previous studies from this laboratory have demonstrated that in the rat uterus the early response to estradiol is characterized by a rapid acceleration of synthetic reactions leading to the accumulation of phospholipid, ribonucleic acid, and protein.[1–6] Levels of puromycin which blocked protein synthesis prevented these responses, suggesting that the primary action of this hormone was to accelerate synthesis of rate-limiting enzymes for these anabolic pathways.[7] Since it has been demonstrated in other biological systems that the synthesis of specific proteins depends on the antecedent synthesis of new RNA species,[8–10] it was of interest to inquire whether or not the early estrogenic response required the synthesis of new RNA.

The recent studies of a number of investigators have documented the ability of actinomycin D to block the DNA-dependent synthesis of RNA in cells and in isolated enzyme systems.[11–14] This paper will describe the use of this agent to prevent RNA synthesis in the uterus of the intact rat. It will be shown that under conditions in which the synthesis of new RNA was blocked by actinomycin D, the early acceleration of phospholipid and protein synthesis, as well as a major portion of the imbibition of water resulting from *in vivo* action of estradiol, failed to occur. The requirement for the synthesis of new RNA in the early estrogenic response is discussed.

Methods.—Female Holtzman rats of the same age and weighing approximately 180 gm were ovariectomized through the dorsal approach and maintained on a diet of Purina dog chow for at least 3 weeks prior to experimentation. Only rats which were ovariectomized on the same day were used in any one experiment.

The rats were injected intraperitoneally with actinomycin D (375 μg in 0.5 ml of 0.154 *M* NaCl) or with saline alone as indicated in the figures. At zero hr 10 μg of estradiol-17β in 1.0 ml of buffered saline containing 1% ethanol[6] or the control solution of buffered saline and ethanol was injected via the tail vein. To assess the *in vivo* synthesis of RNA, protein, and phospholipid, a combination of 25 μc of uridine-H^3 (specific activity = 1.0 mc/0.036 mg) and 6 μc of glycine-$2C^{14}$ (specific activity = 1 mc/12.4 mg) dissolved in 0.5 ml of saline was injected intraperitoneally at 2 and 3 hr. In certain experiments only glycine-$2C^{14}$ was administered.

The rats were killed 4 hr after the injection of hormone by decapitation, and the uteri were removed, stripped of accessory fat, and weighed on a Roller-Smith torsion balance. The uteri were then homogenized in cold distilled water, and the resulting dispersion was treated with 4% perchloric acid (PCA) to remove the acid-soluble fraction. The sedimented tissue residue was washed twice with 4% PCA and then was extracted successively with 90% ethanol, absolute ethanol, and twice with anhydrous ethyl ether. The combined ethanol-ether extracts, making up the crude lipid fraction, were counted in a liquid scintillation counter.

The tissue residue remaining after ethanol-ether extraction was suspended in 0.01 *M* Tris (trishydroxymethylaminomethane) buffer at pH 7.4 and incubated at 37°C for 30 min with 100 μg of RNAse. The reaction mixture was treated with 4% PCA to obtain the RNAse-released material; this fraction was counted for H^3 content in a liquid scintillation counter. The results were expressed as counts per min (CPM) per uterus.

The PCA-insoluble residue was washed successively with 90% ethanol, absolute ethanol, and twice with anhydrous ethyl ether. The residual protein was spread on aluminum planchets with the aid of a small amount of formic acid, and after drying it was counted in a gas flow counter

for C^{14} content. The data on the protein residues are expressed as CPM per mg protein after correction for self-adsorption.

Results.—The effect of actinomycin D on RNA synthesis in the rat uterus: In a preliminary experiment two levels of actinomycin D (125 μg and 375 μg per rat) were administered to test the ability of this agent to block the *in vivo* incorporation of uridine-H^3 into RNA of the rat uterus during the final 2 hr of a 4-hr estrogen treatment. The higher dose inhibited uridine incorporation 90 per cent, whereas the lower dose was slightly less effective. Subsequent trials showed that, within the limits of measurements, the higher dose of actinomycin D blocked RNA synthesis immediately. Since the rats showed no signs of physical difficulty in the 4-hr test period, the 375 μg dose of actinomycin D was used in all subsequent experiments.

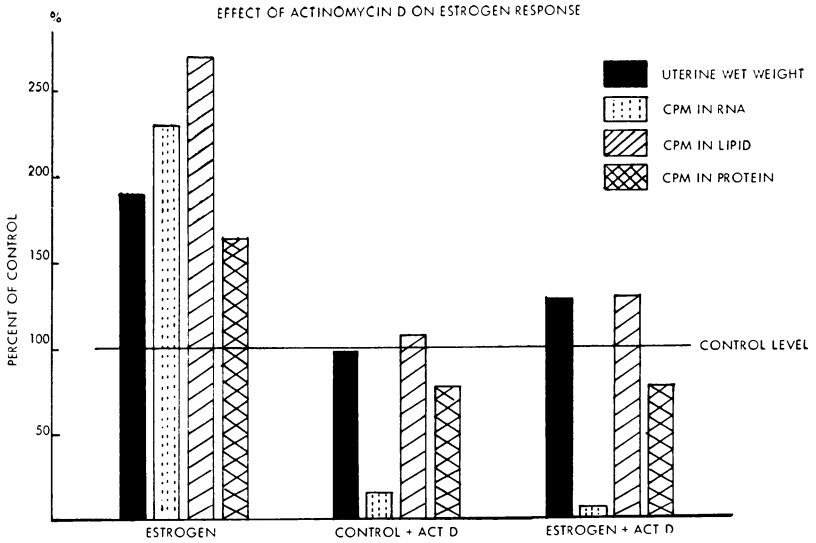

FIG. 1.—Effects of actinomycin D on the early estrogenic response. Groups of 3 rats were injected intraperitoneally with 375 μg of actinomycin D or control solution 30 min prior to the administration of 10 μg of estradiol or control solutions. At 2 hr and again 3 hr after the hormone treatment 25 μc of uridine-H^3 and 6 μc of glycine-$2C^{14}$ were injected intraperitoneally. Four hr after the hormone treatment, the rats were killed and the uteri removed and analyzed for the wet weight, CPM of uridine-H^3 incorporated into RNA, and CPM of glycine-$2C^{14}$ incorporated into protein and mixed lipid fractions. Data are expressed as per cent of values obtained for control uteri which were: wet weight = 71 mg; CPM in RNA per uterus = 986; CPM per mg protein = 81; CPM in lipid fraction per uterus = 235.

Figure 1 shows the data from an experiment in which the effects of actinomycin D were studied in both control and estrogen-treated rats; this experiment typified six successive experiments done at different times and with different groups of ovariectomized rats. It is apparent from the data that actinomycin D was equally effective in blocking RNA synthesis in the uteri of both control and estradiol-treated rats; thus, the usual stimulatory effect of estradiol on RNA synthesis was completely eliminated. Experiments described elsewhere had shown that there is a small amount of labeling of RNA in a mammalian cell which is insensitive to the action of actinomycin D. This is confined to the 4S RNA fraction (transfer

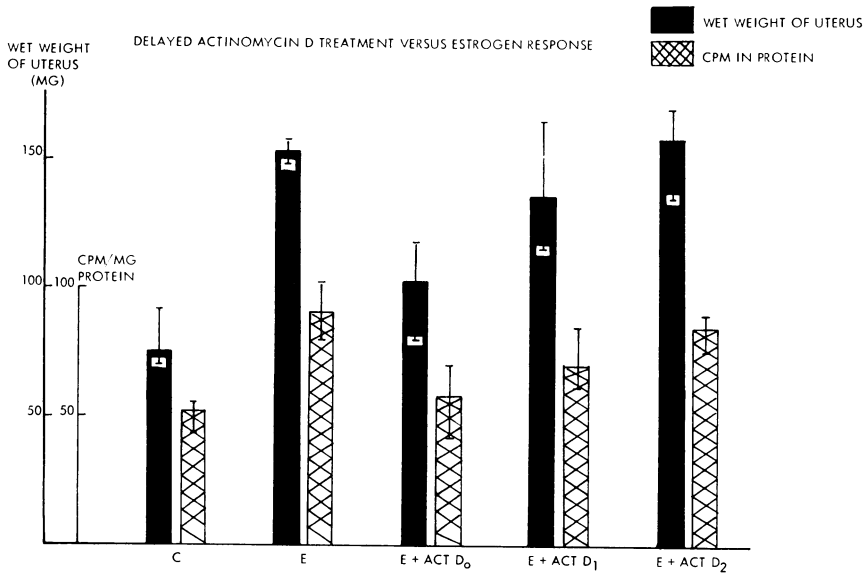

FIG. 2.—Effects of the delayed administration of actinomycin D on the early estrogenic response. Groups of 4 rats each were given intraperitoneal injections of 10 μg estradiol solution and, where indicated, of 375 μg of actinomycin D as follows: simultaneously with estradiol administration (E + Act D_0); 1 hr after estradiol administration (E + Act D_1); and 2 hr after estradiol administration (E + Act D_2). At 2 hr and again 3 hr after the hormone treatment, 6 μc of glycine-$2C^{14}$ was injected intraperitoneally. Four hr after the hormone treatment, the rats were killed, the uteri removed and analyzed for the wet weight, CPM of glycine-$2C^{14}$ incorporated into protein. Data are expressed as mg wet weight/uterus, CPM/mg protein.

RNA) and very likely involves the turnover of the terminal portion of the nucleic acid which accepts an activated aminc acid.[14]

The effect of actinomycin D on the acceleration of protein synthesis by estradiol: As previously shown, estradiol treatment *in vivo* incites a prompt acceleration of protein synthesis;[6] Figure 1 confirms this effect. The accelerating action of the hormone on protein synthesis is, however, prevented by a level of actinomycin D which simultaneously blocks RNA synthesis. On the other hand, actinomycin D appeared to have little or no effect on the basic process of protein synthesis which is in progress, since it caused only a slight decrease in the incorporation of glycine-$2C^{14}$ into the protein of the control uteri. Accordingly, the acceleration of protein synthesis by the hormone appears to require the synthesis of new RNA.

The effect of actinomycin D on the acceleration of lipid synthesis by estradiol: Previous studies demonstrated that estradiol treatment produced a prompt increase in the synthesis of both neutral lipids and phospholipids.[1] In the present experiments the mixed lipid fraction was counted to determine the amount of glycine-$2C^{14}$ which had been incorporated. It had been shown earlier that this incorporation correlated with conversion of glycine to ethanolamine and serine of the phospholipid fraction.[4] Estradiol treatment increased the incorporation of glycine-$2C^{14}$ into the lipid fraction to 270 per cent of the control value (Fig. 1). While treatment with actinomycin D did not significantly affect incorporation of glycine-$2C^{14}$ into the lipid fraction in the control uteri, it prevented approximately 90 per cent of the acceleration caused by estradiol.

The effect of actinomycin D on the imbibition of water: A characteristic effect of estradiol on the rat uterus is the imbibition of water during the first 4 hr of treatment (Fig. 1). In the absence of hormone, actinomycin D caused no alteration in the wet weight of the control uteri. In combination with estradiol, actinomycin D limited the imbibition of water to approximately 30 per cent of that obtained with the hormone alone. This fraction of the response was insensitive to actinomycin D, even when this agent was administered 30 min prior to the injection of estradiol.

The effect of delayed administration of actinomycin D: In two experiments actinomycin D was administered at different times after the initiation of the hormone treatment to test for any periods of unusual sensitivity. As shown in Figure 2, the administration of actinomycin D 2 hr after the hormone had no effect on the imbibition of water by the uteri in the standard 4-hr test period. Therefore, it appears that the process which supports water imbibition is induced during the first 2 hr of hormone action.

On the other hand, the acceleration of protein synthesis by the action of the hormone was a continuing process requiring an actinomycin D sensitive step. A similar result was found for the estrogenic acceleration of phospholipid synthesis.

Discussion and Summary.—A previous report from this laboratory showed that the early estrogenic acceleration of RNA and phospholipid synthesis, as well as the estrogen-induced imbibition of water, could be prevented by levels of puromycin which simultaneously blocked protein synthesis *in vivo*.[7] It was concluded from these data that the early anabolic effects of this hormone depend on protein synthesis, and that this process is the amplifier for the initial hormone interaction with a specific acceptor in the uterine cell.

In the present study, a level of actinomycin D which blocked RNA synthesis effectively *in vivo* also prevented the estrogenic acceleration of phospholipid and protein synthesis. Since the actinomycin D did not affect seriously the basic process of protein synthesis in control uteri during the test period, it would appear that the acceleration of this process as well as the acceleration of lipid synthesis awaited the information contained in the newly synthesized RNA. The observation in these studies that a portion of the water imbibition was insensitive to actinomycin D, yet was sensitive to puromycin,[7] suggests that a part of the information (i.e., RNA) for the hormone response is already available to the protein synthetic machinery of the control uteri, which can, in turn, be activated by the hormone. In accord with this concept, Gorski and associates have recently found that estradiol causes a striking increase of RNA polymerase activity in the nuclei during the first hr of hormone treatment, whereas a number of other anabolic enzymes were found to respond later. The induction of RNA polymerase activity, however, was prevented by puromycin action.[16] In work to be reported elsewhere, it has been shown in centrifugation experiments that estradiol caused an immediate and striking labeling of all classes of RNA.[15]

The summation of these findings prompts the tentative conclusion that estradiol, on engaging a specific receptor system in the uterus, stimulates the synthesis of certain proteins necessary for the induced synthesis of new RNA; the newly synthesized RNA, in turn, supplies the protein-synthesizing machinery with the information essential to the amplification of the initial hormone response.

The authors wish to thank Mrs. Kathleen Deighton for her excellent and cooperative assistance in these experiments.

* This work was supported by grant CA 01897-11 from the U.S. Public Health Service and by funds from the Alexander and Margaret Stewart trust.

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*THE PARTICULATE NATURE OF NONCHROMOSOMAL GENES IN CHLAMYDOMONAS**

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Communicated by M. Demerec, June 7, 1963

The existence of stable genetic determinants not carried on chromosomes and occurring in a wide variety of organisms is well documented and has been summarized in a number of reviews.[1] In most of these studies, the evidence is derived from formal genetic analysis, demonstrating that the pattern of segregation of the mutant phenotype among the progeny of crosses cannot be attributed to chromosomal segregation, whether normal or aberrant. These findings have aroused much speculation, the essence of which can be summarized in terms of two alternative hypotheses: (1) that the mutant phenotypes result from mutations of primary genetic information carried elsewhere than on a chromosome;[1, 2] or (2) that the mutant phenotypes result from metabolic events which shift a series of interlocking reactions from one steady-state level to another, producing an altered phenotype not resulting from any change in primary genetic information.[3]

These alternative views are similar to those that faced Mendel and other analysts of heredity at the turn of the century: Are genetic determinants particulate, discrete units? Do they persist generation after generation whether expressed or not?