*ESTROGEN-INDUCED SYNTHESIS OF A SPECIFIC UTERINE PROTEIN**

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Use of inhibitors of protein synthesis has revealed that the early metabolic responses to estrogen depend on synthesis of protein. Puromycin- and cycloheximide-sensitive changes in the metabolism of lipid, RNA, and glucose are seen in the first hour after treatment with estrogen,[1-3] and before estrogen stimulates over-all synthesis of protein.[2] The implications of these earlier studies are clear: (1) in less than 1 hr, estrogen induces the synthesis of protein essential to the development of the hormone response; (2) the newly made protein(s) is small in amount, contributing little to the picture of net protein synthesis.

This paper presents direct evidence for the presence of such a protein in rat uteri stimulated with estradiol.

Experimental Procedure.—Ten Holtzman rats, 21–23 days old and weighing about 50 gm, were used in each experimental group. Estradiol-17β (5 μg in 0.5 ml 0.154 *M* NaCl) or saline was injected intraperitoneally 0.5, 1, 2, or 4 hr before killing. The excised uteri were placed in 3 ml of Eagle's HeLa tissue culture medium containing H^3-leucine (60 μc, 1 c/mmole, Schwarz) or C^{14} reconstituted protein hydrolyzate (40 μc, 80–180 mc/mmole, Schwarz). The uteri were incubated at 37°C for 1 hr under an atmosphere of 95% O_2–5% CO_2. In some experiments actinomycin D (200 μg in 0.5 ml saline, 4 mg/kg body weight) was injected 30 min before the injection of estradiol.

For *in vivo* labeling H^3-leucine (50 μc/0.25 ml dose, total dose 150 μc, 750 mc/mmole) was injected intraperitoneally three times, 0.5, 1, and 1.5 hr after estradiol. The animals were killed 2 hr after receiving the injection of estradiol.

The uteri were homogenized in 0.5–1.0 ml of 1.5×10^{-3} *M* disodium ethylenediaminetetraacetate (Na_2 EDTA). After centrifuging at 20,000 $\times$ *g* for 30 min, the residue was discarded. The protein in 25 μl of the supernatant was determined by the Lowry procedure.[4] To another 25-μl sample, 0.1 ml of serum was added as a protein carrier. The protein was precipitated with 7% trichloroacetic acid (TCA), washed successively with 5% TCA, absolute ethanol, 2:1 chloroform-ethanol, and ether. The residue was then dissolved in hydroxide of hyamine, and the H^3 or C^{14} content was assayed in a scintillation counter.

A 50-μl sample of supernatant was placed into each slot of a 13% starch-gel block,[5] made with 0.033 *M* Tris (trishydroxymethylaminomethane), 0.010 *M* boric acid, 0.0015 *M* Na_2 EDTA buffer, pH 8.6. At completion of the electrophoretic run of 8–9 hr at 6 v/cm, the gel was sliced lengthwise and the lower half was placed in a solution of amido black 10 B in 5% acetic acid for several hours. Unbound dye and unincorporated labeled amino acids were removed from the gel with an electrophoretic decolorizer. The gels were photographed and sectioned into 1.7-mm strips. Each section was placed in a scintillation counting vial with 97% formic acid to dissolve the starch. The formic acid was evaporated and hydroxide of hyamine added. The samples were counted in a scintillation counter, and the data were plotted as cpm versus distance from the slot in cm. There was no quenching of radioactivity due to the starch or dye in the samples.

Results.—Estrogen induction of in vitro protein synthesis: Starch-gel electrophoresis separates the soluble uterine proteins into a large number of distinct zones (Fig. 1). Figures 2 to 5 depict the labeling patterns of the soluble uterine proteins from whole uteri incubated with labeled amino acids after 0.5, 1, 2, or 4 hr of *in vivo* estradiol treatment. The total radioactivity of Zone A (Fig. 1), which migrated 6.6–6.8 cm from the origin, increased markedly after estradiol treatment with a mean of 180 per cent and a range of 107–273 per cent above the control values in ten

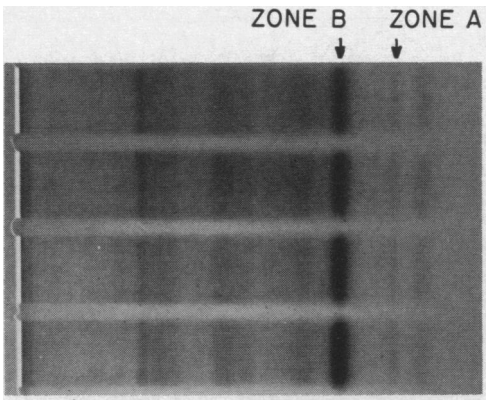

FIG. 1.—Pattern of starch-gel electrophoresis of soluble uterine proteins in Tris-EDTA-boric acid buffer. The proteins migrate from the origin on the left, toward the anode on the right. The specific estrogen-induced protein is located in Zone A. Zone B contains serum albumin which enters the uterine tissues during estrogen treatment. Compare control uterine proteins (*top electrophoretic separation*) to uterine proteins from 4-hr estradiol-treated animals (*bottom*).

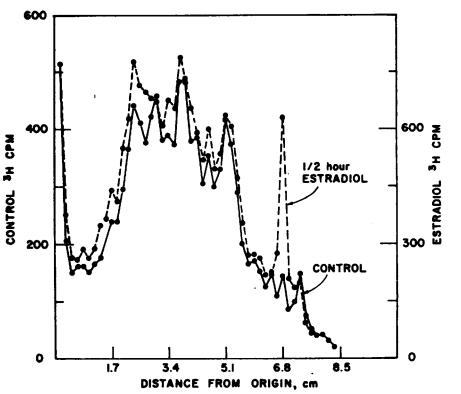

FIG. 2.—Labeling pattern of soluble uterine proteins separated by starch-gel electrophoresis 0.5 hr after an *in vivo* extradiol treatment, followed by a 1-hr incubation in Eagle's medium containing 60 μc of H^3-leucine. The incorporation of H^3-leucine into the uterine protein was stimulated above control values only in those samples containing Zone A.

experiments. The effect on the protein of Zone A was apparent at the earliest time analyzed (Fig. 2), although no other protein-containing band showed changes in the first 2 hr after estradiol treatment. The increased incorporation in Zone A was not due to a specific effect on leucine, since the incorporation of C^{14} amino acids from the protein hydrolyzate increased to the same extent (Fig. 3). The incorporation of amino acid into all uterine proteins has been shown to be greater by 4 hr; nevertheless, the specific effect of estradiol on the protein of Zone A is still detectable (Fig. 5). The other soluble proteins were synthesized in the same relative ratios as in the control tissue.

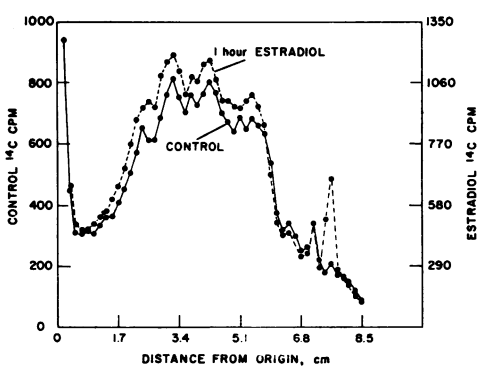

FIG. 3.—Labeling pattern of soluble uterine proteins separated by starch-gel electrophoresis after a 1-hr *in vivo* estradiol treatment, followed by a 1-hr incubation in Eagle's medium containing 40 μc of C^{14} reconstituted protein hydrolyzate. Only the incorporation in Zone A is greater than in the controls.

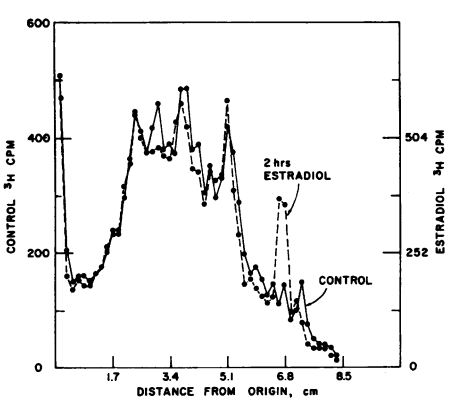

FIG. 4.—Labeling pattern of soluble uterine proteins separated by starch-gel electrophoresis after 2 hr of *in vivo* estradiol treatment, followed by a 1-hr incubation in Eagle's medium containing 60 μc of H^3-leucine.

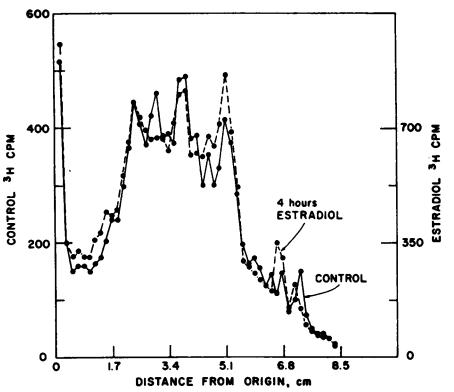

FIG. 5.—Labeling pattern of soluble uterine proteins separated by starch-gel electrophoresis after a 4-hr *in vivo* estradiol treatment, followed by a 1-hr incubation in Eagle's medium containing 60 μc of H^3-leucine. The incorporation into all proteins from estradiol-treated animals was 60% greater than in the controls.

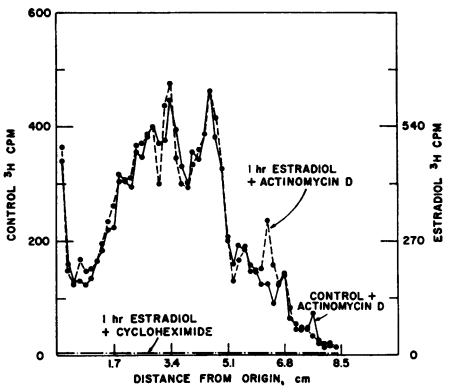

FIG. 6.—Labeling pattern of the soluble uterine proteins after separation by starch-gel electrophoresis. Before the 1-hr *in vivo* estradiol or saline treatment, the animals received 200 μg of actinomycin D for 30 min. The uteri were incubated for 1 hr in Eagle's medium containing 60 μc H^3-leucine. A third group of uteri from animals pretreated for 1 hr with estradiol were incubated in Eagle's medium containing 60 μc H^3-leucine and 10 μg of cycloheximide per ml of Eagle's medium.

Effects of actinomycin D and cycloheximide: To determine whether the synthesis of the specific, estrogen-affected protein associated with Zone A was under transcriptional or translational control, actinomycin D was administered 30 min before injecting the estradiol. The 4 mg/kg dose was much higher than levels usually used *in vivo* to produce complete inhibition of uterine RNA synthesis.[7]

However, in eight experiments in the presence of actinomycin D, the incorporation of labeled amino acids into the induced protein was increased by a 1-hr estrogen treatment to 104 per cent above the control value with a range of 48–247 per cent (Fig. 6). These values are approximately half of the increase due to estrogen usually seen at this time (see above). Complete inhibition of the estrogen-induced protein did not occur even with actinomycin D added to the incubation medium (20 μg/ml). Examination of the specific activity of the total soluble fraction indicated that actinomycin D depressed incorporation of H^3-leucine into all the soluble proteins by 30 per cent.

The presence of cycloheximide (10 μg/ml) in the incubation medium blocked the incorporation of H^3-leucine into all of the proteins, thus verifying that the radioactivity of Zone A was in fact in newly synthesized protein.

Estrogen stimulation of protein synthesis in vivo: The labeling pattern of the soluble uterine proteins after administration of estradiol and H^3-leucine *in vivo* shows that estrogen greatly increased the incorporation of amino acid into Zone A, 7.7 cm from the origin (Fig. 7). Under *in vivo* conditions, estradiol also increased the amount of radioactivity in a second region, Zone B, 6.2 to 6.7 cm from the origin (Fig. 7). The radioactivity associated with Zone B evidently results from the increased uptake of labeled serum albumin by the estradiol-stimulated uterus. The identity of Zone B as serum albumin was supported by finding that in this

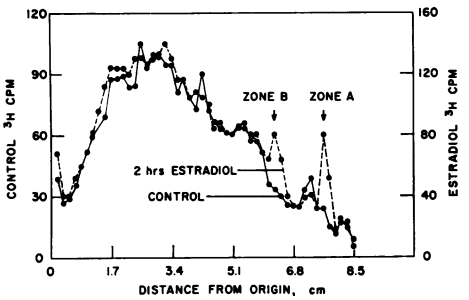

FIG. 7—Labeling pattern of the uterine proteins after *in vivo* administration of H^3-leucine. Estradiol-17β was administered 2 hr before killing. H^3-leucine was administered at 0.5, 1.0, and 1.5 hr after the estrogen. Zone A, which contains the estrogen-induced protein, migrated 7.7 cm from the origin; and Zone B, containing serum albumin, migrated 6.2–6.7 cm from the origin.

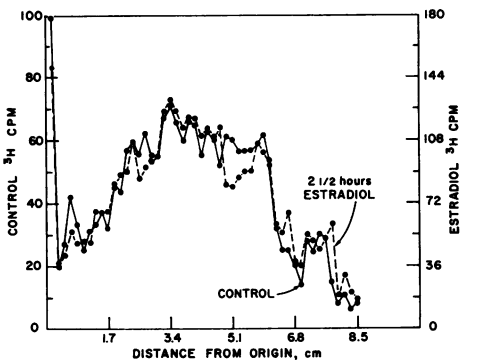

FIG. 8.—Labeling pattern of the soluble proteins of the ileum after *in vivo* administration of H^3-leucine and estradiol. A zone with similar mobility as Zone A of the uterine proteins is located 7.2 cm from the origin.

buffer system the albumin of rat serum had the same electrophoretic mobility as Zone B. Also, the staining intensity of Zone B was increased in estradiol-treated animals without any increase in radioactivity after incubation with labeled amino acids *in vitro* (Figs. 2–5). The increase in staining was seen only in Zone B and never in the other zones.

The estrogen effect on the protein of Zone A was highly specific for rat uterus, since estradiol did not influence the pattern of incorporation of leucine into the soluble proteins of liver and ileum. As shown in Figure 8, ileum has proteins with the same mobilities as those in the uterine Zones A and B, but they were unaffected by the hormone.

Discussion.—Within 30 min after its administration, estradiol stimulated incorporation of amino acids into a uterine protein of a particular electrophoretic zone. The incorporation undoubtedly represented *de novo* synthesis of protein, since it was blocked when cycloheximide was present in the incubation medium. H^3-leucine and a C^{14} reconstituted protein hydrolyzate both served as precursors for the protein, indicating that incorporation did not reflect end-group labeling with leucine. These data are presented as direct evidence that early estrogen action involves the synthesis of a specific protein.

While numerous workers have observed hormonal stimulation of the synthesis of specific enzymes, the responses reported usually involve a number of enzymes.[6] The work reported here demonstrates a specific synthesis induced by estrogen prior to a general effect on protein synthesis. Since only 20–40 per cent of the total uterine protein was in the soluble fraction analyzed in these experiments, it is possible that proteins present in the insoluble fractions may also be affected.

The failure of actinomycin D to block effectively the synthesis of the estrogen-induced protein during a time when RNA synthesis was inhibited 80–90 per cent can be interpreted in two ways: (1) The induction may be under translational control and RNA synthesis may not be directly involved in the synthesis of this protein. The observed partial decrease in the synthesis of the estrogen-induced protein could be due to the turnover of the messenger RNA, or to secondary effects

of actinomycin D as reflected by the 30 per cent decrease in general protein synthesis. (2) An alternative explanation for our data is that actinomycin D is less effective in suppressing the RNA synthesis at the gene locus controlling the estrogen-induced protein as compared to other loci. Different experimental approaches will be necessary to resolve this problem.

Hamilton[8] has reported that actinomycin D failed to suppress all of the estrogen-induced increase of over-all protein synthesis. In his study, however, RNA synthesis was reduced only to 30–50 per cent of the control levels. The work of Ui and Mueller[7] indicates that under conditions of 90 per cent inhibition of RNA synthesis with actinomycin D, no estradiol-accelerated synthesis of protein occurs. Neither the present evidence suggesting translational control, nor our previous data showing that the estrogenic stimulation of RNA synthesis and RNA polymerase depend on protein synthesis,[2, 3] support the recent suggestion of Talwar *et al.*[9] that estrogens directly derepress RNA synthesis. Instead, it would appear that the estrogen-receptor complex[10] may be involved in controlling the activity of a specific protein-synthesizing system. Ames and Hartman have suggested one possible mechanism for translational regulation.[11] However, the complexity of the protein-synthesizing system prevents even speculation as to the regulation of the estrogen-induced protein.

The function of the induced protein in the uterine response to estrogen raises several interesting, unsolved questions. As yet, we have no evidence that increased incorporation is reflected by accumulation of the protein. The labeled zone is closely associated with the dye-absorbing band, but visual evaluations of the staining of the band revealed no increases in the first 4 hr after injection of estrogen. However, the electrophoretic band may include several proteins which could mask quantitative changes in a minor, estrogen-sensitive component. Whatever the situation, it seems probable that the synthesis of this protein is a key step in the action of estrogen.

Summary.—The effects of estrogen on the incorporation of labeled amino acids into soluble uterine proteins was studied by starch-gel electrophoresis. Early induction of the synthesis of a single protein occurred within 30 min after estrogen treatment and prior to the over-all stimulation of protein synthesis. The synthesis of this estrogen-induced protein was still demonstrable but at lowered levels in the presence of actinomycin D, suggesting that either its synthesis is under translational control or that RNA synthesis in the specific gene locus for this protein is not effectively suppressed by actinomycin D.

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[1] Mueller, G. C., J. Gorski, and Y. Aizawa, these PROCEEDINGS, **47**, 164 (1961).

[2] Noteboom, W. D., and J. Gorski, these PROCEEDINGS, **50**, 250 (1963).

[3] Gorski, J., and M. C. Axman, *Arch. Biochem. Biophys.*, **105**, 517 (1964).

[4] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

[5] Smithies, O., *Biochem. J.*, **71**, 585 (1959).

[6] Segal, H. L., and Y. S. Kim, *J. Cell. Comp. Physiol.*, **66**, Suppl. 1, 11 (1965).
[7] Ui, H., and G. C. Mueller, these PROCEEDINGS, **50**, 256 (1963).
[8] Hamilton, T. H., these PROCEEDINGS, **51**, 83 (1964).
[9] Talwar, G. P., S. J. Segal, A. Evans, and O. W. Davidson, these PROCEEDINGS, **52**, 1059 (1964).
[10] Toft, D., and J. Gorski, *J. Cell Biol.*, **27**, 107A (1965).
[11] Ames, B. N., and P. E. Hartman, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 28 (1963), p. 349.