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

By Gerald C. Mueller, John Gorski,† and Yoshio Aizawa‡

McArdle Memorial Laboratory, University of Wisconsin Medical School

Communicated by Henry Lardy, December 7, 1960

Previous studies from this laboratory on the mechanism of estrogen action have demonstrated that a single dose of estradiol causes a rapid acceleration of synthetic reactions which lead to the accumulation of phospholipids, ribonucleic acids, and proteins in the uterus of the ovariectomized rat.[1–6] In those metabolic pathways which have been investigated in cell-free systems (i.e., one carbon metabolism and protein synthesis), the early acceleration by estrogen was correlated with a simultaneous increase in the activities of certain participating enzymes.[7,8] These data suggested the possibility that the metabolic alterations in general may result from hormonally induced increases in enzyme levels.

Since attempts to activate hypothetical proenzymes were uniformly negative, experiments were initiated to assess the role of protein synthesis in the estrogen-induced responses. The recent report of Yarmolinsky and de la Haba[9] demonstrating that the antibiotic, puromycin, inhibited protein synthesis in rat liver enzyme preparations prompted its use in the present studies. This paper will demonstrate that with the proper *in vivo* administration of puromycin, protein synthesis can be blocked almost entirely in the rat uterus without inhibition of phospholipid or ribonucleic acid synthesis in this tissue. Under conditions in which protein synthesis was blocked by puromycin, the early acceleration of phospholipid and ribonucleic acid synthesis as well as the characteristic imbibition of water resulting from the *in vivo* action of estradiol failed to occur. In accordance with these findings, the requirement for protein synthesis in the early estrogenic response is discussed.

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

Puromycin, 15 mg suspended in 0.5 ml of buffered saline (0.154 *M* NaCl in 0.04 *M* NaH_2PO_4-Na_2HPO_4 buffer at pH 7.4) was injected intraperitoneally at 0, 1, 2, and 3 hours.§ Controls received only the buffer. Estradiol-17β, 10 μg in 1.0 ml of buffered saline containing one per cent ethanol, or the buffered saline-ethanol control solution was injected via the tail vein at zero hour as indicated. Glycine-2-C^{14}, 0.25 or 0.50 mg per dose, (specific activity 1.19 μcuries/μmole) dissolved in buffered saline was injected intraperitoneally as indicated at 0, 1, 2, and 3 hours in two trials and at 0 and 2 hours in one trial. In other experiments, inorganic orthophosphate-P^{32}, 600 or 750 μcuries dissolved in 0.5 ml of 0.154 *M* NaCl (5.9×10^3 μcuries/μmole), was injected intraperitoneally at zero time.

The animals were killed at 4 hours; the uteri were removed, stripped of fat and connective tissue, and weighed on a Roller-Smith Torsion balance. The uteri were then placed in ice-cold five per cent trichloroacetic acid (TCA) and either pulverized from the frozen state in a stainless steel mortar cooled with liquid air or homogenized in an all glass homogenizer in cold five per cent TCA. Tissue residues were washed four times in ice cold five per cent TCA to remove the acid-soluble fraction. The lipid fraction was extracted at room temperature by successive washes of 80 per cent ethanol, 100 per cent ethanol, chloroform-ethanol (2:1), ether and ether.[1]

In experiments in which glycine-2-C^{14} was the radioactive precursor, the nucleic acid–protein residues were plated and counted in an internal gas flow counter. The results were expressed as counts per minute (C.P.M.) per mg of residue after correction for self absorption. Previous

studies have demonstrated that over 90 per cent of the radioactivity was due to the label in the protein of this residue.[6]

The isolation of the nucleic acid purines was carried out according to the procedure of LePage.[10] For this purpose, the residues from two uteri were combined and extracted with four per cent perchloric acid at 90° for 30 minutes. The mixed purine bases which were separated from the extract by adsorption and elution on ashort column of Dowex 50 (H^+ form) were purified to constant specific activity by chromatography on paper first in a solvent system consisting of isopropanol, isobutyric acid, H_2O, and 30 per cent NH_4OH (60:25:14:1) and then in a system of N-butanol and H_2O (86:14). Horizontal suspension of the paper during the chromatography gave the optimal separation of the bases. After elution of the bases from the final chromatograms, aliquots were plated and counted in an internal gas flow proportional counter and the quantity of adenine determined by the absorption at 260 mμ.

In experiments in which inorganic orthophosphate-P^{32} was the radioactive precursor, the lipid fraction remaining after evaporation of the combined organic solvent extracts was chromatographed on silicic acid–impregnated paper according to the method of Marinetti by using diisobutyl ketone, acetic acid, and H_2O (40:25:5) as the solvent system.[11] The phosphotides were located by radioautography. The ethanolamine phospholipid spot was eluted, aliquots were taken for counting in an end window counter, and the phosphate was determined by the method of Chen.[12] Results are expressed as C.P.M. per μg of P.

The labeling of the ribonucleic acid fraction was determined on the ribonucleotides isolated from the alkaline degradation of the RNA. For this purpose, the pooled protein-nucleic acid residues from two uteri were hydrolyzed in 0.2 *N* NaOH at 37° for 14 hours. After precipitation of the acid-insoluble residue with cold ten per cent perchloric acid, the nucleotides were adsorbed on charcoal (nuchar C) and then eluted with 2.5 per cent pyridine in 50 per cent ethanol as described by Tsuboi.[13] The chromatographic isolation of the AMP and CMP was achieved by chromatography on paper at 5°C using isobutyric acid and 0.4 *M* NH_4OH (75:25) as the solvent system. The nucleotide areas were eluted and aliquots were counted in an end window counter and assayed for AMP and CMP by ultraviolet absorption. Results were expressed as C.P.M. per μmole of nucleotide.

Results.—The effect of puromycin on the incorporation of glycine-2-C^{14} into uterine protein: In order to establish the *in vivo* effectiveness of puromycin as a blocking agent for protein synthesis, control and puromycin-treated rats were injected with glycine-2-C^{14}, and the amount of isotope incorporated into uterine protein during a four-hour period was determined. In preliminary experiments with varying doses and schedules, it was found that the intraperitoneal injection of 15 mg of puromycin each hour gave approximately a 90 per cent inhibition of the incorporation of glycine-2-C^{14} into uterine protein of the control rats over the test period (Fig. 1). This dose of puromycin was similarly effective in blocking protein synthesis in the estrogen-treated rats. It was also observed in the course of these experiments that the synthesis of uterine proteins had fairly well resumed by two hours after a single 15-mg dose, thus necessitating the hourly injections of puromycin. The experiments were confined to four-hour periods so as to provide adequate time for highly significant estrogen responses and yet not so long as to incur secondary toxicities resulting from the blockage of protein synthesis with puromycin.

In another report,[14] it will be shown that this dose of puromycin is equally effective in inhibiting the incorporation of glycine-2-C^{14} into proteins of the liver but was less effective in the heart, thymus, and kidney (64, 51, and 60 per cent inhibition, respectively).

The effect of puromycin on the incorporation of glycine-2-C^{14} into nucleic acid purines: In contrast to its action on protein synthesis, puromycin had little or no

effect on the incorporation of glycine-2-C^{14} into the adenine of the mixed nucleic acids of the control uteri (Fig. 2). On the other hand, the characteristic acceleration of nucleic acid purine labeling during the first four hours of estradiol action was completely prevented by the simultaneous administration of puromycin; it is of interest that the incorporation rate actually remained at the control level throughout the four-hour period.

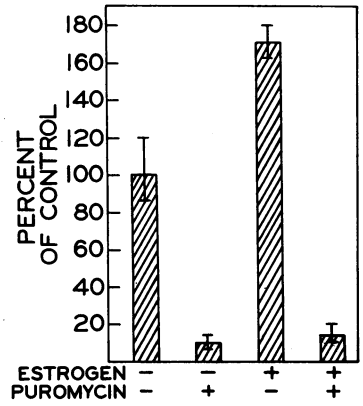

FIG. 1.—Glycine-2-C^{14} incorporation into protein–nucleic acid residue of rat uteri. Each bar is the average of 3 (−puromycin) or 4 (+puromycin) rat uteri. 15 mg of puromycin (+) or a control solution (−) were injected intraperitoneally at 0, 1, 2, and 3 hr. 0.5 mg of glycine-2-C^{14} (6.2 μC) were injected intraperitoneally at 0, 1, 2, and 3 hr. 10 μg of estradiol-17β (+) or a control solution (−) were injected via the tail vein at 0 hr. Rats were killed at 4 hr and the uteri removed. The protein–nucleic acid residue was obtained by procedures described under methods. The residue was plated and counted and the results calculated as C.P.M. per mg protein residue after correction for self absorption. The data in this figure are expressed as per cent of the control value which was 428 C.P.M./mg of protein residue. Brackets indicate range.

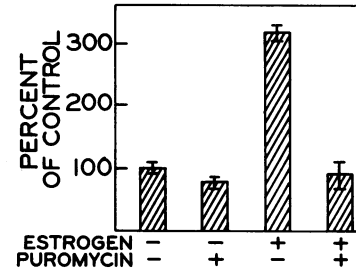

FIG. 2.—Glycine-2-C^{14} incorporation into nucleic acid adenine. Same experiment as in Figure 1. Protein-nucleic acid residues were hydrolyzed in 4 per cent PCA at 90°C. Adenine was isolated by a combination of column and paper chromatography as described under methods. Specific activity was determined by counting C^{14} activity and measuring adenine by its ultraviolet absorption. Results in this figure are expressed as per cent of the control value which was 16 C.P.M./μmole adenine.

The influence of puromycin and estradiol on the incorporation of radioactive phosphorus into ribonucleic acids of the rat uterus: In experiments in which inorganic orthophosphate-P^{32} was used as the radioactive precursor, it was demonstrated that puromycin treatment resulted in an actual increase of the labeling of RNA (Fig. 3) of the control tissues. However, as in the case of glycine-2-C^{14}, the acceleration of RNA synthesis by estradiol was largely prevented by the action of puromycin, and the labeling was essentially the same as seen in the puromycin-treated control rats. The reason for the increased labeling in the uterine RNA of the control rats which were treated with puromycin alone is not apparent at this time.

Alterations of phospholipid synthesis by estradiol and puromycin treatment in vivo: In previous experiments with surviving uterine segments from rats pretreated with estradiol, it has been demonstrated that the synthesis of phospholipids is an unusually sensitive indicator of early estrogenic action.[1] This observation has been reconfirmed and extended in the present *in vivo* study. In Figure 4, the results of an experiment are shown in which the incorporation of inorganic orthophosphate-P^{32} into the ethanolamine phosphatide fraction has been measured *in vivo* over the first four hours following the administration of estradiol. It is evident that the

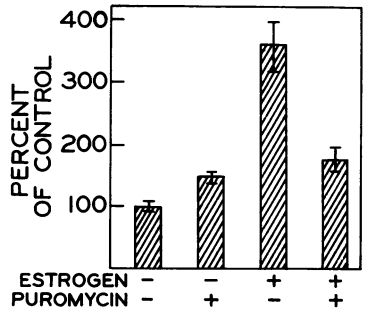

FIG. 3.—The incorporation of orthophosphate-P^{32} into uterine RNA. Each bar is the average of four rat uteri. 15 mg of puromycin or a control solution was injected intraperitoneally at 0, 1, 2, and 3 hr. 622 μC-inorganic orthophosphate-P^{32} was injected intraperitoneally at 0 hr. 10 μg estradiol-17β or a control solution was injected via the tail vein at 0 hr. Rats were killed and uteri removed at 4 hr. RNA was degraded with alkali, and the 2', 3' AMP isolated by paper chromatography as described under methods. Results are expressed as per cent of the control value which was 2360 C.P.M./μmole of AMP.

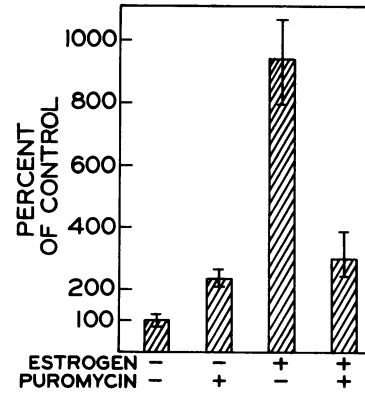

FIG. 4.—The incorporation of orthophosphate-P^{32} into phosphoethanolamine. Same experiment as Figure 3. Lipid fraction was chromatographed on silicic acid–impregnated paper as described under *Methods*. Results are expressed as per cent of the control value which was 151 C.P.M./μg of ethanolamine phosphorus.

single dose of estradiol increases the incorporation of radioactive phosphorus into this phospholipid fraction over 800 per cent.

As in the case of the labeling of the RNA, the administration of puromycin to the control rats also stimulated the labeling of the ethanolamine phosphatide fraction significantly; as yet, this stimulation is not understood. However, the administration of puromycin to rats treated simultaneously with estradiol prevented almost entirely the estrogen acceleration of the phospholipid labeling. Instead, the amount of P^{32} incorporated into the ethanolamine phosphatide fractions was of the same order as obtained in the control rats treated with puromycin alone.

Effect of estradiol and puromycin on the uterine wet weight: A characteristic action of estrogens widely used for their assay is the hormonally induced imbibition of water in the uterus of the overiectomized rat. In Figure 5, the interference with

this phenomenon by puromycin treatment is shown. While puromycin treatment had little or no effect on the wet weight of the control uteri, the estradiol-induced inhibition of water was almost completely blocked. This experiment is representative of seven separate experiments.

Discussion.—The data reported above demonstrate that puromycin at the proper dosage is a highly effective agent for blocking protein synthesis *in vivo* in the uterus of both control and estrogen-treated rats. Under the same conditions, the synthesis of nucleic acid purines from radioactive glycine and the incorporation of inorganic orthophosphate-P^{32} into the uterine RNA and phospholipids of puromycin-treated rats continued at the control level or was stimulated somewhat. Thus, while protein synthesis was interrupted, a number of other basic synthetic processes continued at near the normal level. However, in all cases, the early and characteristic accelerating action of estradiol on phospholipid and RNA synthesis was prevented by the simultaneous action of puromycin *in vivo*. Accordingly, one must conclude as a minimum that the expression of early estrogen action is highly dependent on some puromycin-sensitive process.

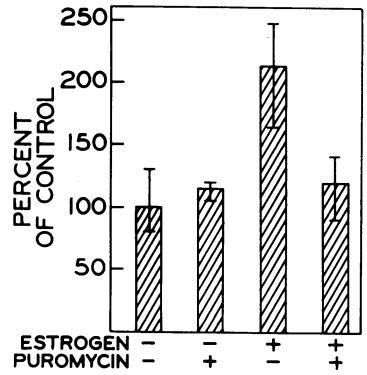

FIG. 5.—The effect of puromycin on the imbibition of water in uteri of estrogen-treated rats. Same experiment as in Figure 1. Each bar is the average wet weight of four uteri 4 hr after initiation of the *in vivo* treatments. Results are expressed as per cent of the average control value which was 65 mg per uterus.

The observation of Yarmolinsky and de la Haba[9] that puromycin blocks effectively the step involving the transfer of the activated amino acid from the soluble ribonucleic acid to the formation of a new peptide linkage in the ribosomes suggests that the puromycin-sensitive step necessary for estrogen action is protein synthesis. The data reported in this paper are in accord with the selective inhibition of protein synthesis by puromycin and point to the usefulness of this agent in the investigation of other metabolic problems.

The observation that the characteristic imbibition of water into estrogen-treated uteri can also be blocked by puromycin treatment *in vivo* argues decisively against any direct alteration of cellular permeability by the hormone. The results, instead, suggest that even this aspect of the estrogen response is dependent on a puromycin-sensitive reaction which appears to involve protein synthesis. The conclusion that estrogens do not act by altering permeability had been arrived at previously by Halkerston *et al.*[15] through direct studies on uterine permeability.

In these studies, it would be of interest if some estrogen action could be demonstrated which was not blocked by puromycin. So far, evidence of such a process has not been forthcoming. Similarly, all attempts to demonstrate an estrogen-sensitive uterine transhydrogenase[16, 17] and hence its participation in the estrogenic response in the rat uterus have been negative.

In a previous discussion of the mechanism of action of estrogens, it has been pointed out that an understanding of the problem awaits both the identification of the primary molecular interaction of the hormone with the cell receptor and the

elucidation of the system whereby the effects of this interaction are amplified.[2] The data presented here suggest that the process of protein synthesis is the cellular amplifier and that the estrogen response is expressed through the production of rate-limiting enzymes. Accordingly, attention is now directed to a study of the manner in which estrogens accelerate protein synthesis in the uterus. Whether estrogens act primarily to activate or inhibit some existing enzyme which in turn contributes to the improvement of the intracellular environment for protein synthesis in general or whether it induces the *de novo* synthesis of such an enzyme remains to be ascertained.

Summary.—The administration of puromycin to ovariectomized rats blocked uterine protein synthesis *in vivo* without inhibition of phospholipid or ribonucleic acid synthesis. Under the same conditions, puromycin treatment also prohibited the early acceleration of phospholipid and ribonucleic acid synthesis by estradiol and prevented the hormone-induced imbibition of water in the uterus. The essentiality of protein synthesis for early estrogen action is discussed.

The authors wish to acknowledge with gratitude the excellent technical assistance of Mr. Donald M. Fechner.

* This work was supported by grant (CY-1897-C7) from the United States Public Health Service and by funds from the Alexander and Margaret Stewart trust.

† Postdoctoral Fellow of the National Cancer Institute, U.S. Public Health Service.

‡ On leave from the Department of Pharmacology, Tokyo Jikei University School of Medicine, Tokyo, Japan.

§ The authors wish to express their deep gratitude to Dr. Nestor Bohonas and the Lederle Company for the generous supplies of puromycin used in this work.

[1] Aizawa, Y., and G. C. Mueller, *J. Biol. Chem.* (in press).

[2] Mueller, G. C., A. M. Herranen, and K. F. Jervell, *Recent Progr. Hormone Research*, **14**, 95 (1958).

[3] Jervell, K. F., C. R. Diniz, and G. C. Mueller, *J. Biol. Chem.*, **231**, 945 (1958).

[4] Herranen, A., and G. C. Mueller, *J. Biol. Chem.*, **233**, 369 (1956).

[5] Mueller, G. C., and A. M. Herranen, *J. Biol. Chem.*, **219**, 585 (1956).

[6] Mueller, G. C., *J. Biol. Chem.*, **204**, 77 (1953).

[7] Herranen, A., and G. C. Mueller, *Biochim. Biophys. Acta*, **24**, 233 (1957).

[8] McCorquodale, D. J., and Mueller, G. C., *J. Biol. Chem.*, **232**, 31 (1958).

[9] Yarmolinsky, M. B., and G. L. de la Haba, these PROCEEDINGS, **45**, 1721 (1959).

[10] LePage, G. A., *Cancer Res.*, **13**, 178 (1953).

[11] Marinetti, G. V., J. Erbland, and J. Kochen, *Federation Proc.*, **16**, 837 (1957).

[12] Chen, P. S., Jr., T. Y. Toribara, H. Warner, *Anal. Chem.*, **28**, 1756 (1956).

[13] Tsuboi, K. K., *Arch. Biochem. Biophys.*, **83**, 445 (1959).

[14] Gorski, J., Y. Aizawa, and G. C. Mueller, submitted for publication.

[15] Halkerston, I. D. K., J. Eichhorn, M. Feinstein, E. Scully, and O. Hechter, *Proc. Soc. Exptl. Biol. Med.*, **103**, 796 (1960).

[16] Talalay, P., and H. G. Williams-Ashman, these PROCEEDINGS **44**, 15 (1958).

[17] Villee, C. A., and D. D. Hagerman, *J. Biol. Chem.*, **233**, 42 (1958).