Uterine Phosphamidase in Sex Hormone-Treated and Deciduomata-Bearing Ovariectomized Rats.* (26334)

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The enzyme phosphamidase hydrolyzes the N-P bond of amides of phosphoric acid. The naturally occurring phosphamides such as creatine and arginine phosphates, however, are not subject to phosphamidase hydrolysis *in vivo,* but are dephosphorylated by the action of the ATP-transphorylase system. Therefore, no specific metabolic role can be ascribed to this enzyme at present.

Previous reports concerning uterine phosphamidase have been limited to histochemical study of the enzyme in biopsies of human endometrium taken during the menstrual cycle(1,2). The present investigation was undertaken to localize the sites of phosphamidase activity in the uterus of the rat, to determine the amount quantitatively, and to ascertain whether variations in enzyme activity followed simulation of the uterus by steroid sex hormones or occurred during the deciduomal reaction.

Materials and methods. Ten to 24 days following bilateral ovariectomy, 95 virgin female Wistar rats, 90 to 120 days of age, were divided into the following 8 groups of 8 to 20 animals each. Rats in each group were treated as described below: (1) controls—no further treatment, (2) injected with 2 μg of estradiol benzoate† 2 days before sacrifice, (3) injected with 10 μg of estradiol benzoate 2 days before sacrifice, (4) injected daily with 2 mg of progesterone‡ for 3 days before sacrifice. In the remaining 4 groups the uteri were sensitized with progesterone as above and were then traumatized on the fourth day by passing a sterile silk thread longitudinally through the uterine lumen. The hormone injections were continued and the animals were sacrificed at 2, 4, 6 and 8 days after traumatization. Each dose was dissolved in 0.1 ml of a mixture of sesame and olive oils and was injected subcutaneously.

Animals were killed by decapitation after sublethal administration of chloroform. Uteri were removed and divided into 3 portions. The largest was weighed to the nearest 0.1 mg and homogenized in 9 times its weight of distilled water in a cold Potter-Elvehjem apparatus. The second portion was also weighed, then dried to determine the amount of tissue residue. The third portion was fixed in cold ethanol for 24 hours and paraffin sections were subsequently prepared for histochemical localization of the enzyme.

Phosphamidase activity was determined quantitatively by a modification of the method of Holter and Li(3) which utilizes liberation of inorganic phosphorus from N-(p-chlorophenyl) - diamidophosphoric acid. Duplicate 0.3 ml aliquots of the 10% tissue homogenate were added to 0.1 ml of a solution containing 11 mM of substrate,§ 20 mM of sodium hydroxide, 19.2 mM of sodium acetate and 60.8 mM of acetic acid per liter. pH of the substrate solution was 4.6. After 2 hours incubation at 40°C enzyme activity was arrested by adding 2 ml of 10% trichloroacetic acid. Liberated phosphorus was measured by the method of Fiske and Subbarow(4). The results have been expressed as micrograms of phosphorus liberated/10 mg of dry tissue/2 hours incubation at 40°C (Table I).

In addition, sites of enzyme activity were

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† Progynon—Schering Corp., Bloomfield, N. J.

‡ Progesterone— B and B Medical Supply Co., Cincinnati, Ohio.

§ N-(p-chlorophenyl)-diamidophosphoric acid was purchased from Dajac Laboratories of Borden Co., Philadelphia, Pa. as "p-chloroanilido-phosphonic acid synthesized by the method of Otto." It has been shown that the former rather than the latter compound is obtained by Otto's procedure. Both, however serve satisfactorily as substrates for demonstration of phosphamidase activity.

TABLE I. Uterine Phosphamidase Activity in Sex Hormone-Treated and Deciduomata-Bearing Ovariectomized Rats.

Treatment	No. of assays	Phosphamidase activity*
None	9†	16.9 ± 1.1‡
2 μg estradiol benzoate, 1 dose 2 days before sacrifice	8	15.0 ± .9
10 μg *idem*	8	12.3 ± 1.5
2 mg progesterone, daily for 3 days before sacrifice	8†	9.8 ± 1.6
Deciduomata, 2 days after uterine traumatization	9	20.7 ± 2.8
Idem, 4 days after	10	51.7 ± 4.0
" , 6 " "	9	42.4 ± 4.3
" , 8 " "	8	26.8 ± 3.4

* μg of phosphorus/10 mg of dry tissue/2 hr incubation at 40°C.

† Tissues from 2 or 3 uteri were pooled for each assay.

‡ Mean ± S.E.

determined histochemically in the tissue sections by the method of Gomori(5) as modified by Meyer and Weinmann(6).‖

Observations. Enzyme activity in the animals treated with 2 μg of estrogen did not differ significantly from that in the untreated castrates. On the other hand, the level of activity was significantly depressed in the rats treated with 10 μg of estrogen ($p<0.02$) and with progesterone ($p<0.01$)(Table I). In the uteri containing deciduomata, in all instances phosphamidase activity was greater than in the animals treated with progesterone alone ($p<0.01$ in each case). Progressively higher levels were observed through the fourth day following traumatization—when a peak of more than 5 times the activity in untraumatized progesterone-treated animals was reached. Thereafter there was a progressive decrease in deciduomal enzyme activity.

Histochemical results were not uniform. As previously experienced with the technic (5,6,7), staining varied within individual sections and in consecutive sections taken from the same tissue specimen. The reason for this variability lies in the fact observed by the present authors(8) that less than 8% of the original phosphamidase activity of fresh tissue survives in tissue sections prepared in the prescribed manner. Because of this problem the sites of enzyme activity described below are composite patterns derived through microscopic study of a number of sections from each specimen.

In both the untreated and hormone-treated castrates uterine phosphamidase was confined to the luminal and glandular epithelia of the endometrium and tended to be more concentrated at either the apical or basal pole. There was an inverse relationship between overall intensity of cytoplasmic and nuclear staining. In the deciduomata, on the other hand, in addition to the epithelial activity described above, phosphamidase was present in the differentiating cells of the endometrial stroma. Unlike the epithelial staining, staining in the stromal cells was generally uniform throughout the cytoplasm. However, the same inverse relationship between cytoplasmic and nuclear activity was present. Furthermore, a distinct zonation of activity was apparent in the deciduomata themselves, especially on fourth and sixth days after traumatization. That is, in the deciduomal cells closest to the uterine lumen there was intense cytoplasmic activity, but little or no nuclear reaction. Progressing centrifugally a gradual reversal of staining was observed so that at the periphery of the deciduoma nuclear activity was greatly increased and cytoplasmic activity sharply reduced or absent.

Discussion. Both Remotti(1) and Oehlert, *et al.*(2) observed a pattern of phosphamidase activity in human endometrium which differed from that seen in the rat. They found that during the menstrual cycle enzyme activity was present in the nuclei of the stromal cells as well as throughout the epithelial cells. There was a gradual increase throughout the follicular phase of the cycle which reached a peak during the secretory phase. Late in the latter phase phosphamidase activity declined and disappeared during menstruation. In the rat, on the other

‖ In a personal communication Dr. Julia Meyer suggested further modifications of Gomori's method which have been incorporated. The incubation solution used contained 4.41 mM of substrate (supplied through the courtesy of Dr. Meyer), 3.5 mM of lead nitrate, 45.0 mM of malaic acid and 34.8 mM of sodium hydroxide per liter, and had a pH of 4.1.

hand, both estrogen and progesterone decrease the activity below that present in the uterus of the castrate and the enzyme does not appear in the endometrial stroma unless the latter is first traumatized. It is evident that there is a considerable species difference both in distribution of phosphamidase in the endometrium and in its hormonal regulation.

Meyer and Weinmann(9,10,11) have studied the distribution of phosphamidase in a number of rat organs and have correlated the enzyme activity with the physiological processes known to occur in the same sites. They have found phosphamidase to be associated with (1) active transport and other types of physicochemical work, (2) synthesis of nonprotein substances in both exocrine and endocrine glands, and (3) cellular differentiation. These processes are known to take place in various component tissues of rat endometrium and are largely dependent upon stimulation by estrogen and progesterone. Since the present experiments have demonstrated that both these hormones depress phosphamidase in the castrate rat uterus, the hypothesis of Meyer and Weinmann cannot be supported in this instance. In the deciduomal reaction, on the other hand, where there is a centrifugally progressive differentiation of stromal cells into decidual-like cells, phosphamidase activity approximates that which would be expected on the basis of the experience of Meyer and Weinmann. The present observations reemphasize the paucity of information concerning the metabolic role of phosphamidase and the need for further study of its activity in other tissues and organs.

Summary. The sites of phosphamidase were localized histochemically and amount of enzyme activity measured quantitatively in the uteri of untreated, estrogen- and progesterone-treated, and deciduomata-bearing ovariectomized rats. Enzyme activity was found in the lumenal and glandular epithelia of the endometria of all the animals studied. In addition, phosphamidase was present in the differentiating stromal cells in the uteri in which the deciduomal response had been elicited. Estrogen in high dosage and progesterone depressed the enzyme activity compared with that in untreated castrates. During the development of deciduomata the enzyme level rose to over 5 times that in animals treated with progesterone but not traumatized.

1. Remotti, G., *Clin. Ostet. e Ginec.* (Milano), 1954, v78, 1079.

2. Oehlert, G., Neumann, K., Hansmann, H., *Arch. f. Gynak.*, 1954, v184, 414.

3. Holter, H., Li, Si-Oh, *Compt. Rend. Lab. Carlsberg, Ser. Chim.*, 1951, v27, 393.

4. Fiske, G. H., Subbarow, Y., *J. Biol. Chem.*, 1925, v66, 375.

5. Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 407.

6. Meyer, J., Weinmann, J. P., *J. Histochem. and Cytochem.*, 1955, v3, 134.

7. Gomori, G., *Microscopic Histochemistry,* Univ. Chicago Press, 1952, 196.

8. Atkinson, W. B., Herbener, G. H., *Proc. 1st Internat. Cong. Histochem. and Cytochem.,* in press.

9. Meyer, J., Weinmann, J. P., *J. Histochem. and Cytochem.*, 1957, v5, 354.

10. ———, *Am. J. Anat.*, 1957, v101, 461.

11. ———, *J. Invest. Dermat.*, 1957, v29, 393.

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Antitumor Activity of 1-β-D-Arabinofuranosylcytosine Hydrochloride. (26335)

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In this laboratory as well as others, investigations of purine and pyrimidine derivatives as potential antimetabolites which might inhibit *in vivo* biosynthesis of DNA and/or RNA have lead to development and clinical study of such compounds as 6-mer-