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ROLE OF ESTROGEN-INDUCED UTERINE PEROXIDASE IN THE METABOLISM OF ESTRADIOL *IN VIVO*

H. S. KEEPING and P. H. JELLINCK
Department of Biochemistry, Queen's University, Kingston, Ontario K7L 3N6, Canada

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SUMMARY

The nature of the steroids present in the uteri of immature rats and in those of animals pretreated with estrogen which contained peroxidase was examined 10 min and 2 h after the *in vivo* administration of 0.25 μg of [^{3}H]-estradiol. Only unchanged estradiol and some estrone were detected in the uteri of both groups of rats and most of the radioactivity was released into the medium after incubation with *p*-hydroxymercuribenzoate (PHMB), a sulfhydryl-blocking reagent. Similarly, an 80-fold excess of diethylstilbestrol (DES) injected together with [^{3}H]-estradiol prevented the uterine uptake of the ^{3}H-labelled compound and also displaced it from the uteri of both control and estrogen-pretreated rats. The concentration of estrogen-receptor in the cytosol of immature and estradiol-pretreated rats was determined by two different methods to show that the receptor had been replenished in the uteri of rats given estrogen 20 h before the experiment to induce peroxidase. These results indicate that even though uterine peroxidase can catalyze the metabolism and covalent binding of estradiol to protein *in vitro*, it is unlikely to limit the duration of estrogen action in the intact animal by this mechanism. Other possible roles for the induced enzyme are considered.

INTRODUCTION

It is now well established that a peroxidase that catalyzes the metabolism and covalent binding of estradiol to protein *in vitro* can be induced in the uteri of immature rats by physiological doses of estradiol or gonadotrophin [1–3]. It is also known that the ability of immature rat uteri to retain [^{3}H]-estradiol previously incorporated either *in vivo* or *in vitro* is destroyed by treating the tissue with various sulfhydryl-blocking reagents [4]. It was therefore decided to determine whether the [^{3}H]-estradiol present in the uterus of rats containing the induced peroxidase would still be removed during incubation with *p*-hydroxymercuribenzoate (PHMB) or whether the estrogen would be retained as a result of covalent binding to uterine cell constituents. This would help to establish whether the induced enzyme was able to metabolize estradiol in the intact animal and therefore played a role in limiting estrogen action by inactivating estradiol [5, 6]. These experiments were repeated using an excess of diethylstilbestrol instead of an organic mercurial to displace [^{3}H]-estradiol by competition from its high-affinity binding site and, in both cases, the extracts were analyzed for metabolites.

EXPERIMENTAL

Materials. Crystallized and freeze-dried bovine serum albumin, estrone, estradiol-17β, diethylstilbestrol (DES), *p*-hydroxymercuribenzoate (sodium salt), dithiothreitol, Dextran (av. mol. wt 83,000) and Norit A were purchased from Sigma Chemical Co., St Louis, MO, U.S.A., 2,4-dichlorophenol and guaiacol from Eastman Kodak Co., Rochester, NY, U.S.A., and protamine sulphate (USP injection, without phenol preservative) from Eli Lilly Co., Toronto, Ontario. [4-^{14}C]-estradiol (40 mCi/mmol) from Schwarz/Mann, Orangeburg, NY, U.S.A., and [6,7-^{3}H]-estradiol (40–60 Ci/mmol) from New England Nuclear Corp., Boston, MA, U.S.A. were shown by thin-layer chromatography (t.l.c.) to be >98% pure radiochemically. The ^{14}C-labelled steroid was diluted with carrier to a specific radioactivity of 2–3 mCi/mmol and kept at 4°C in the dark as a stock solution in ethanol (1 mg/ml). All solvents were redistilled and the diethyl ether used for extraction was free of peroxides.

Treatment of rats. Immature female Holtzman rats (20–22 days old) primed with a subcutaneous injection of estradiol (5 μg in 0.2 ml sesame oil) on day 1, were given a further injection of estradiol (1 μg in 0.5 ml of 0.9% NaCl) or saline on day 5. [^{3}H]-estradiol (50 μCi in 0.25 μg) in 0.9% NaCl (0.5 ml) containing 3% (v/v) ethanol was then administered s.c. 20 h later with or without added diethylstilbestrol (20 μg). This last step was omitted in the experiments to determine cytosol receptor concentration or peroxidase activity.

Retention of [^{3}H]-estradiol by uteri. In general, the experimental protocol of Jensen *et al.* [4] was followed. The rats, anaesthetized with CO_2, were killed by cervical dislocation; the uteri were dissected free of adhering fat and mesentery and slit longitudinally before being weighed. Five uterine horns (2.5 uteri)

were then stirred at 37°C for 30 min or 1 h in 400 ml Krebs–Ringer–Henseleit (KRH) glucose buffer (pH 7.3) [7] alone or containing 1 mM *p*-hydroxymercuribenzoate (PHMB) and the eluting medium, changed at each time point. It was filtered and samples (1.0 ml) taken for determination of radioactivity by scintillation counting as described previously [3]. The tissue after incubation was removed from the medium, blotted dry and left overnight at 4°C in ethanol (0.5 ml). It was further extracted by grinding with ethanol (final vol: 2 ml) in a glass homogenizer with a Teflon pestle and centrifuged at 1000 *g* for 10 min. Samples (0.2 ml) of the supernatant and of the residual uteri dissolved at 45°C in NCS (Amersham/Searle) tissue solubilizer (2 ml) were then assayed for ^3H. The radioactivity in the tissue is the sum of these two values and is expressed as d.p.m. The same method of incubation and extraction was used in the experiments with DES; however, the sulfhydryl-blocking reagent was omitted from the medium.

Determination of peroxidase activity. A homogenate of the uteri in 5 ml of 0.1 M sodium phosphate buffer, pH 7.0, was diluted to 11 ml with buffer and centrifuged at 100,000 *g* (4°C) for 30 min. The sediment, after washing with buffer, was rehomogenized in the appropriate amount of 1.2 M NaCl to give a 5% (w/v) homogenate and centrifuged again at 100,000 *g* for 30 min. Portions of this uterine extract (0.5 ml) derived from 25 mg wet wt of tissue were then assayed for peroxidase activity by using guaiacol as substrate [8] or by the conversion of [^{14}C]-estradiol to water-soluble products [9].

Examination of uterine extract for [^3H]-estradiol metabolites. The ethanol extract of the uteri was evaporated to dryness under N_2 at 40°C and examined by t.l.c. on silica gel in cyclohexane–ethyl acetate–ethanol (10:9:1 v/v/v) [10] or benzene:ether (6:4 v/v) [11] with estrone, estradiol, 2-hydroxyestradiol and estriol-16α,17β as standards. These were visualized by spraying with dilute Folin–Ciocalteu reagent followed by exposure to ammonia vapor [12]. Sections (2.5 × 0.5 cm) of the chromatogram were scraped into scintillation vials and the radioactivity determined in Aquasol (New England Nuclear Corp.). To confirm the identity of [^{3}H]-estrone and [^{3}H]-estradiol by reverse isotope dilution, the corresponding areas were scraped into test tubes and the radioactive steroids extracted from the silica gel with 10 ml of chloroform–methanol (1:1 v/v). The extracts were centrifuged at 1000 *g* for 10 min and the supernatant solution decanted into pre-weighed tubes which were evaporated to dryness under N_2 at 25°C after the addition of 10–15 mg of non-radioactive carrier steroid. Each sample was dissolved in boiling acetone (1–2 ml) and, after reducing the volume to one half, it was recrystallized by the dropwise addition of *n*-pentane until the solution just became turbid. The crystals were removed by centrifugation (1000 *g*, 10 min) and the mother liquor decanted into preweighed tubes for evaporation to dryness under N_2. Both were further dried over $CaCl_2$ *in vacuo* to ensure constant weight and the specific activity (^{3}H d.p.m./mg) for the crystals and the material in the mother liquor determined after each of 3 crystallizations.

Determination of cytosol binding sites by [^3H]-estradiol exchange assay. Two different methods were used. In the assay of Chamness *et al.*[13] as modified by Zava *et al.*[14] the cytosol receptor, from which unbound steroid had been removed by dextran–charcoal, was precipitated with protamine sulphate before incubation with various concentrations of [^{3}H]-estradiol with or without a 100-fold excess of unlabelled estradiol (for determination of non-specific binding). In the other assay [15] the cytosol receptor was incubated in solution with [^{3}H]-estradiol. In both cases, the uteri were frozen over solid CO_2, pulverized with a tissue pulverizer (Thermovac Industries) and further dispersed in buffer with a Brinkman Polytron PT-10-ST at speed 3.5 for three 10 s intervals. All glassware was siliconized by immersion in a 1% (v/v) solution of Siliclad (Becton Dickinson Co., Canadian Laboratory Supplies, Toronto, Ontario) followed by rinsing with glass-distilled H_2O. The protein concentration of the 100,000 *g* supernatant (cytosol) was determined by the method of Lowry *et al.*[16].

RESULTS

The effect of adding *p*-hydroxymercuribenzoate (PHMB) to the incubation medium on the retention of [^{3}H]-estradiol by uteri of immature or estrogen-treated rats given [6,7-^{3}H]-estradiol 2 h before sacrifice is shown in Fig. 1A. In the absence of this sulfhydryl-blocking reagent most of the original ^{3}H-label was retained by the slit uterine horns even after incubation for 1 h, whereas in the presence of PHMB, nearly all the radioactivity was eluted from the tissue and appeared in the medium with both immature and estradiol-treated animals. In both cases, virtually all the ^{3}H in the uteri could be extracted into ethanol and was shown by t.l.c. to be entirely as unchanged estradiol.

Similar results were obtained with PHMB when the animals were killed 10 min after the administration of [^{3}H]-estradiol (Fig. 1B) but in these experiments a larger fraction of the radioactivity remained associated with the tissue in the presence of PHMB and a higher concentration of ^{3}H was found in the uteri of estrogen-treated rats. In both these groups, about 2–3% of the radioactivity in the tissue was shown by t.l.c. to be due to [^{3}H]-estrone and blood was also found to contain this metabolite of estradiol. Greatly decreased amounts of radioactivity were found associated with uteri when an 80-fold excess of DES was administered simultaneously with [^{3}H]-estradiol 2 h before sacrifice (Fig. 2A) and virtually all the ^{3}H was lost from the tissue after 1 h of incubation in KRH glucose buffer (Fig. 2A). The decrease in ^{3}H-uptake by the uteri of both control

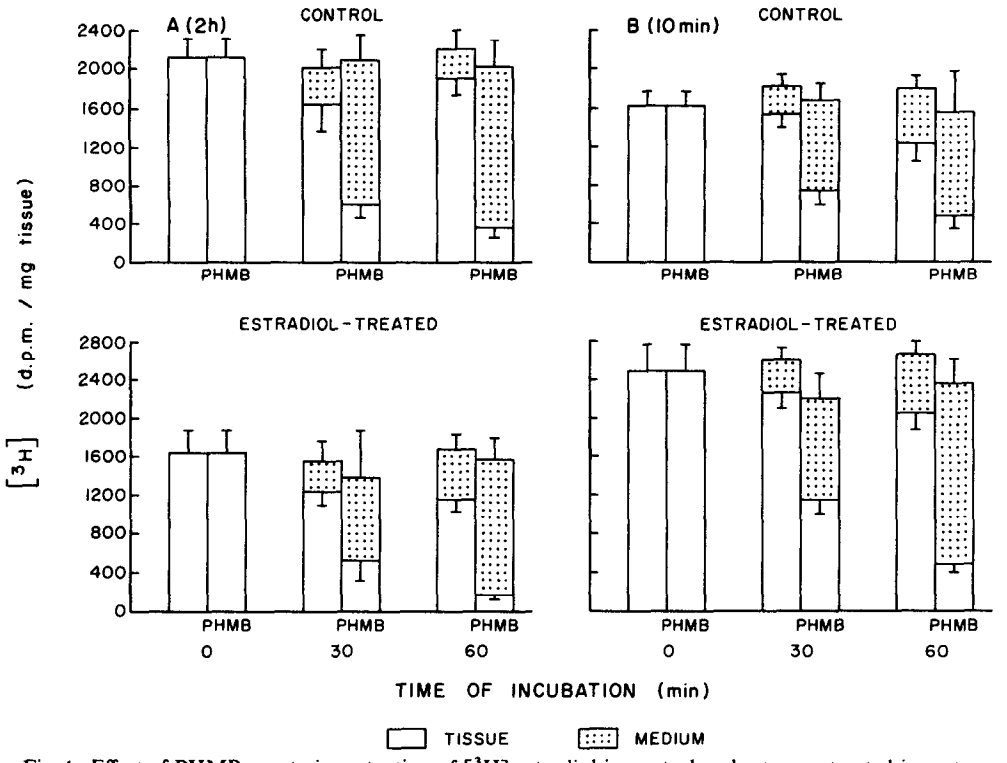

Fig. 1. Effect of PHMB on uterine retention of [^{3}H]-estradiol in control and estrogen-treated immature rats. The animals were given [^{3}H]-estradiol (50 μCi in 0.25 μg) in 0.9% NaCl (0.5 ml) subcutaneously 2 h (A) and 10 min (B) before sacrifice. The estrogen-treated rats had received 1 μg of estradiol s.c. in saline 20 h before the injection of the radioactive steroid. The slit uterine horns were stirred at 37°C in KRH–glucose buffer (pH 7.3) for the times indicated in the presence and absence of PHMB (1 mM). The radioactivity released into the medium and retained by the tissue was determined as described in the text. Each bar represents the range of values from two experiments.

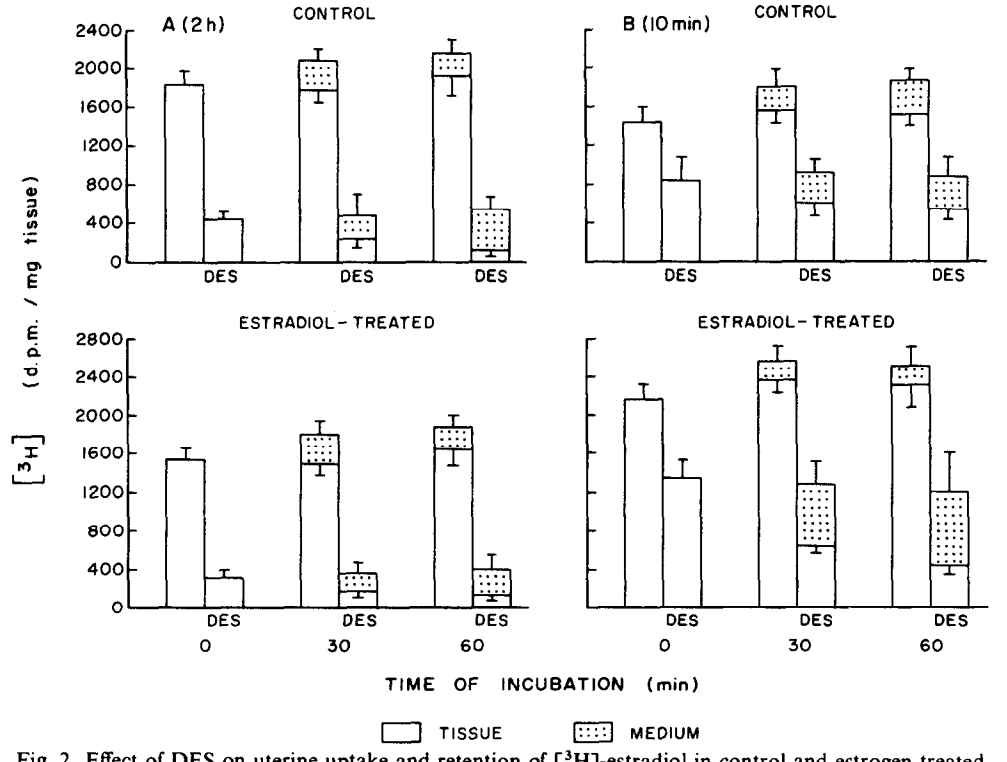

Fig. 2. Effect of DES on uterine uptake and retention of [^{3}H]-estradiol in control and estrogen-treated immature rats. The animals were given [^{3}H]-estradiol mixed with an 80-fold excess of DES (20 μg) 2 h (A) and 10 min (B) before sacrifice. No PHMB was added to the incubation medium. Other conditions as in Fig. 1.

Table 1. Identification of estradiol and estrone from uteri of immature rats by reverse isotope dilution and recrystallization to constant specific activity

	Number of crystallizations	Specific activity (dpm/mg) Estradiol	Estrone
Control			
	1	9378	316
	2	9351	287
	3	9331	281
Estradiol-treated			
	1	11358	348
	2	11313	339
	3	11287	325

[^{3}H]-Estradiol and [^{3}H]-estrone in the ethanol extract of uteri were separated by t.l.c. and eluted for further analysis by reverse isotope dilution as described in the text. The specific activity of the material in the mother liquor also remained constant after recrystallization.

and estradiol-treated animals in the presence of excess DES was less pronounced when the rats were killed only 10 min after the administration of [^{3}H]-estradiol (Fig. 2B). No significant difference in the uterine retention of ^{3}H was found between immature and estrogen-pretreated rats in either the 10 min or 2 h groups of animals. However, as in the experiments with PHMB, only unchanged estradiol was found in the tissue when [^{3}H]-estradiol was administered 2 h before sacrifice whereas both estradiol and estone were present when the animals were killed after 10 min. Confirmation of the identity of these steroids was carried out by reverse isotope dilution and recrystallization to constant specific activity (Table 1).

The presence of peroxidase in the estrogen-treated animals was determined by two different methods and, as expected, an increase in uterine weight was also observed under these conditions (Table 2).

The concentration of estrogen receptor in the uterine cytosol of control and estradiol-treated immature rats was measured by two different methods. Less non-specific binding was observed after precipitation with protamine but good agreement was obtained for the concentration of specific binding sites (Bsp) in the uterus by these two assays (Fig. 3).

In other experiments, using only the highest concentration (20 nM) of [^{3}H]-estradiol needed to saturate the specific binding sites, the total binding capacity of the uterine cytosol before and after treatment with estradiol was determined (Table 3). It is apparent from these results that much of the receptor protein had been replenished 20 h after treatment with estradiol (1 μg).

DISCUSSION

These results clearly demonstrate that, even in the presence of peroxidase, an enzyme capable of converting estradiol to covalently-bound water-soluble products *in vitro* [1–3] only unchanged estradiol and traces of estrone can be found in rat uteri *in vivo* either 10 min or 2 h after the administration of [^{3}H]-estradiol. The findings of Jensen *et al.* [4] that the sulfhydryl-blocking reagents destroy the ability of uteri to retain previously incorporated estradiol was confirmed and PHMB was chosen because it caused a more rapid release of the steroid than either *N*-ethylmaleimide or iodoacetamide [4] and did not inhibit uterine peroxidase [17]. It had been reasoned that if the estrogen-induced uterine peroxidase had the role of limiting estrogen action by metabolizing estradiol, a difference in the distribution of [^{3}H]-estradiol in uterine fractions (water-soluble vs tissue-bound) would be observed between estrogen-treated and control rats, particularly in the presence of PHMB. The experiments in which the ^{3}H-labelled hormone was administered 2 h before examining the uteri for metabolites were repeated allowing only 10 min between injection and sacrifice in case most of the water-soluble products had diffused away from the uteri during

Table 2. Effect of treatment with estradiol on peroxidase activity and uterine weight in immature rats

Treatment	% of added ^{14}C in aqueous medium after extraction with ether	Rate of oxidation of guaiacol (μmole/min per mg tissue)	Uterine wet weight (mg)
Control	6.5 ± 1.7	0.007 ± 0.001	61.8 ± 1.6
Estradiol-treated	43.1 ± 2.5	0.266 ± 0.026	111.4 ± 17.6

Primed immature rats were given estradiol (1 μg in 0.5 ml saline) subcutaneously 20 h before sacrifice. The control rats received vehicle alone. Preparation of uterine extracts and assay of peroxidase activity as described in the text. Results are the mean of 5 experiments ± the standard deviation.

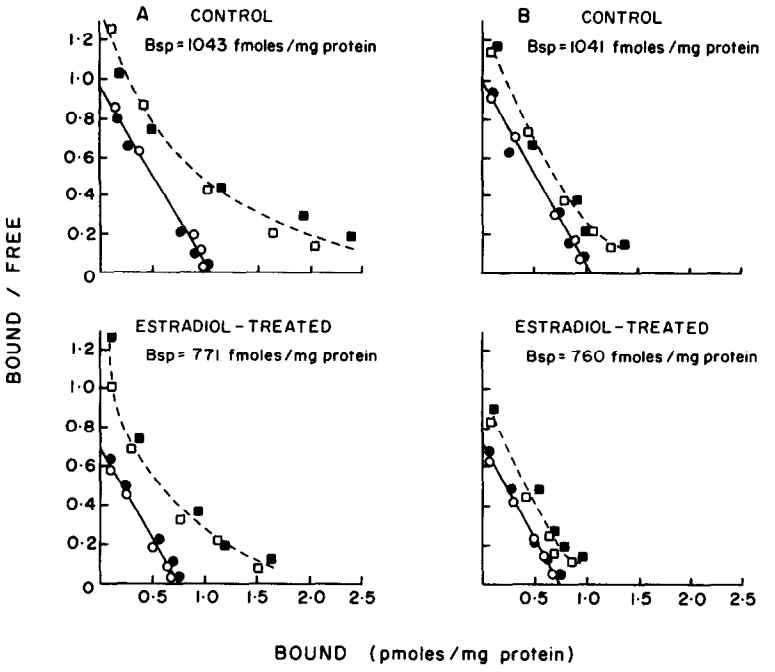

Fig. 3. Scatchard analysis to determine the number of specific sites (Bsp) for estradiol in the uterine cytosol of control and estrogen-treated immature rats. Specific binding (——) of [^{3}H]-estradiol to receptor sites in solution (A) and in a protamine precipitate (B) was determined after subtracting the non-specific binding values from the total amount bound (– – –). The filled (●■) and open (○□) symbols represent values from each of two experiments.

the longer time interval. However, apart from a small difference in pattern of [^{3}H]-estradiol uptake between control and estrogen-treated rats, the results with added PHMB were not altered. Similar findings were obtained when an excess of DES administered *in vivo* was used to displace [^{3}H]-estradiol from its high-affinity uterine binding sites. Although much of the estradiol receptor in the cytosol would be translocated to the nucleus after treatment with estrogen (1 μg), it will have been replenished to normal levels by 20 h [18] and would thus be available to bind [^{3}H]-estradiol (0.25 μg) given subsequently. This was indicated by the retention of [^{3}H]-estradiol during *in vitro* incubation in the absence of PHMB and was confirmed by direct measurement using two different assays. Thus it appears that metabolism of estradiol does not occur even when peroxidase is present or the estrogen is displaced from its high affinity receptor in rat uteri.

There are several possible explanations: Uterine peroxidase has been shown to be localized in reticular membrane-bound vesicles and some regions of the Golgi apparatus [19, 20] and therefore may be sequestered in a compartment of the cell remote from estradiol. Alternatively, the concentration of H_2O_2 required for the reaction may not be in the effective range [17] because of destruction by catalase or different production rates in different subcellular fractions [21].

It appears, therefore, that a role other than to limit the duration of action of estradiol in its target tissue must be considered for the induced peroxidase. It could be involved in the iodination of proteins or other halogenation reactions similar to those described for the thyroid [22] and it is known that peroxidases in association with halides and H_2O_2 exert bactericidal [23] and possibly spermicidal effects [24]. The binding activity of the estrogen receptor

Table 3. Concentration of estrogen receptor in the uterine cytosol of control and estradiol-treated immature rats as determined by two different exchange assays

Assay	Expt. No.	Estradiol binding capacity (pmoles/uterus) Control	Estradiol-treated
Direct exchange	1	1.14	1.63
	2	1.17	1.59
Exchange after precipitation with protamine	1	1.18	1.56
	2	1.16	1.57

Primed immature rats were given estradiol (1 μg in 0.5 ml saline) subcutaneously 20 h before sacrifice. The control rats received vehicle alone. An aliquot (0.2 ml) of the uterine cytosol was incubated with [^{3}H]-estradiol (16 or 20 nM) in the exchange assays and corrected for non-specific binding as described in the text.

is also destroyed by iodination [25] but peroxidase is unlikely to catalyze this reaction without also iodinating estradiol, and it is known that the estrogen-receptor complex is protected from attack by iodine [25]. Another possible role for peroxidase might be to catalyze reactions involved in the cross-linking of tyrosyl residues of uterine proteins and thus produce a more rigid meshwork in the estrogen-stimulated uterus. In this respect, it has been shown that horseradish peroxidase can increase the dityrosine content of collagen [26] and that increased amounts of dityrosine are present in uterine proteins during pregnancy [27]. Furthermore, ovoperoxidase from sea urchin eggs is known to bring about the hardening of the fertilization membrane with tyrosine cross-links [28]. Experiments to test these other possible functions of the induced uterine enzyme are in progress.

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