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ON THE MECHANISM OF THE INCREASE IN PROLYL HYDROXYLASE ACTIVITY IN THE UTERUS OF THE RAT GIVEN ESTRADIOL-17β

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Abstract—The activity of prolyl hydroxylase (EC 1.14.11.2; proline, 2-oxoglutarate dioxygenase) and the protein content of the uterus were significantly increased 4 hr after the administration of a single dose of estradiol-17β to either the immature rat or the adult ovariectomized rat. In contrast to results in the uterus, prolyl hydroxylase activity was decreased in heart, kidney and lung of the immature rat after estradiol-17β. Prolyl hydroxylase activity reached a maximum in 24–32 hr, and subsequently the enzyme activity decreased toward control values. In both animals, there was a 50 per cent reduction in enzyme activity 8–12 hr after reaching maximum activity. The estradiol-17β-induced increase in prolyl hydroxylase activity was blocked by inhibitors of RNA and protein synthesis. In addition, an increase in total enzyme protein was demonstrated using an enzyme immunossay. Although these data provide evidence that *de novo* synthesis of enzyme protein occurs in the uterus after the administration of estradiol-17β, it does not rule out the possibility that other post-transcriptional effects of estradiol-17β play a role in increasing the activity of this enzyme. Efforts to stimulate uterine prolyl hydroxylase activity with either cAMP or dibutyryl cAMP were unsuccessful.

A critical step in the biosynthesis of collagen is the arrangement of the pro α-chains into the triple helical configuration [1]. A necessary requirement for the stability of the triple helix at 37° is the hydroxylation of an appropriate number of prolyl residues of the pro α-chains [2, 3]. Therefore, the enzyme prolyl hydroxylase, which catalyzes the formation of hydroxyproline, is a key enzyme in the control of collagen biosynthesis. However, although prolyl hydroxylase activity is elevated in all tissues which are actively synthesizing collagen [4–8], little is known regarding those factors which modulate the activity of this enzyme.

The administration of estradiol-17β to the immature rat causes a rapid increase in the collagen content of the uterus [9]; this response is associated with a significant increase in prolyl hydroxylase activity [10]. A similar increase in the activity of this enzyme was found in the uterus of the adult ovariectomized rat [10]. In these studies, there was some evidence to suggest that the increase in prolyl hydroxylase activity was due to *de novo* synthesis. In this report, further evidence is presented to support this suggestion.

MATERIALS AND METHODS

Female rats of the Long–Evans strain were used in these experiments.* The immature rats were 20 days of age and weighed approximately 35 g. The ovariectomized rats, with an age variation of no more than 7 days, were castrated when body weight reached 155–160 g and experiments were begun 22 days later using only those animals weighing 200–230 g. Estradiol-17β was dissolved in 5% ethanol in saline and injected intraperitoneally. The uterine horns and cervix of each rat were excised after removing all adhering tissue. Water within the lumen of the uterine horns was expressed and the tissue was blotted before determining the wet weight. In this report, the term 'uterus' refers to the uterine horns and cervix.

Prolyl hydroxylase was extracted from the uterus as follows. The fresh tissue (one to three uteri) was homogenized at 0° with an all-glass homogenizer in 0·05 M sodium cacodylate buffer (pH 7·0), containing 0·2 M NaCl, 10^{-4} M dithiothreitol and 10^{-5} M EDTA. The homogenates were centrifuged at 27,000 *g* for 30 min. The supernatants were assayed for prolyl hydroxylase activity by the tritium release method using [3,4-^{3}H]proline-labeled substrate prepared from granuloma tissue [11]. The granuloma was induced in guinea pigs by subcutaneous injection of carrageenan [12].

Protein concentration was determined on the 27,000 *g* supernatant using the method of Sutherland *et al.* [13] with bovine serum albumin as the standard.

Either 5 mg cyclic adenosine monophosphate (cAMP) or dibutyryl cAMP was administered intraperitoneally in saline at 8-hr intervals; the uteri were removed 8 hr after the second dose. Theophylline (5 mg i.p.) was administered as a single dose on day 1 and as two divided doses on day 2. Estradiol-17β (0·01 μg/day, i.p.) was injected immediately after the first of two daily doses of theophylline. The uteri were removed 48 hr after the first dose of theophylline. The uteri were weighed and the amount of protein and the activity of prolyl hydroxylase were determined as described above.

For the determination of prolyl hydroxylase activity and prolyl hydroxylase-related antigen, the tissues were homogenized at 0° with an all-glass

* Long–Evans rats were purchased from Blue Spruce Farms, Altamont, N.Y.

homogenizer in 0·01 M Tris–HCl buffer (pH 7·4) containing 0·25 M sucrose, 0·1% Triton X-100, 10^{-4} M dithiothreitol and 10^{-5} M EDTA. The homogenates were centrifuged at 27,000 *g* for 30 min, and the supernatants were assayed for prolyl hydroxylase activity by the tritium release method [11] using a [3,4-^{3}H]proline-labeled substrate prepared from 8-day-old chick embryos. The enzyme immunoassay is based on the finding that prolyl hydroxylase is inhibited by the antibody to this enzyme and that enzymatically inactive but immunologically reactive protein can compete with enzyme for antibody binding sites [14]. The enzymatic activity of the extracts was destroyed by heating at 56° for 10 min. However, heating does not destroy the ability of the inactivated enzyme or cross-reacting protein to combine with the antibody directed against the active enzyme. Prolyl hydroxylase, purified from newborn rat skin [15], which had been heated to destroy its enzyme activity, was used as a standard antigen. Therefore, the total amount of antigen in an extract could be expressed in terms of enzyme equivalents. The amount of cross-reacting protein in the extract was calculated by substracting the prolyl hydroxylase content, which was determined before heating, from the total antigen content.

RESULTS

Time course of the increase in activity of prolyl hydroxylase in the uterus of both the immature and ovariectomized rat after the administration of a single dose of estradiol-17β. There is a marked increase in the specific activity and total activity of prolyl hydroxylase in the uterus of the immature rat after the administration of 5 μg estradiol-17β. Maximum activity is observed at approximately 24 hr (Fig. 1). The results in the ovariectomized rat are qualitatively the same, although there appears to be a longer time-lag before specific activity rises (Fig. 2). The administration of estradiol-17β to both the immature and the ovariectomized rat stimulates the synthesis of total protein in the uterus. The wet weight of the immature uterus was significantly increased 4 hr after estradiol-17β, reached a maximum at 24 hr, and had almost returned to control value at 48 hr. The wet weight of the uterus of the ovariectomized rat was highest 4 hr after estradiol-17β and declined to control value at 48 hr (data not shown).

Time course of the decrease in activity of prolyl hydroxylase in the uterus of the immature and ovariectomized rat after the administration of a single dose of estradiol-17β. The prolyl hydroxylase activity decreases slowly in the uterus of the immature rat after reaching a maximum 24 hr following the administration of a single dose of estradiol-17β (Fig. 3). The gradual decline in enzyme activity is paralleled by a decrease in total protein. In the ovariectomized rat, there is a sharp initial decrease in prolyl hydroxylase activity (from 32 to 40 hr) followed by a slow decline toward the control value (Fig. 4). In both the immature and ovariectomized rat, there was a 50 per cent reduction in enzyme activity 8–12 hr after reaching a maximum activity. When these data are compared to the decrease of enzyme activity observed in other experiments (Figs. 1 and 2), it is obvious that there

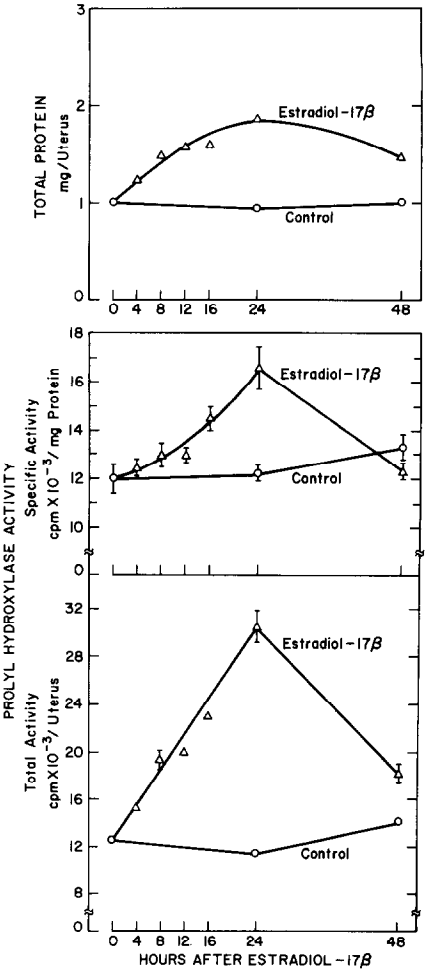

Fig. 1. Time course of the increase in prolyl hydroxylase in the uterus of the immature rat after the administration of estradiol-17β. Groups of 20-day-old rats were given either 5 μg estradiol-17β or an equal volume of vehicle (5% ethanol in saline) intraperitoneally. Each point is the mean value ± S.E.M. of the determinations on at least three 27,000 *g* supernatants, each prepared from three uteri. The standard error bar (I) is absent if this value falls within the area of the symbol for the mean.

can be considerable biological variation in the half-life of prolyl hydroxylase.

Effect of cAMP and dibutyryl cAMP on prolyl hydroxylase activity in the uterus of the immature rat. It has been reported that after the administration of cAMP or dibutyryl cAMP, the wet weight and the activity of phosphofructokinase of the uterus increased significantly. These effects were augmented when either nucleotide was given in combination with theophylline [16, 17]. These treatments have been assessed in the immature rat for their effect on uterine wet weight and on the activity of prolyl hydroxylase in the uterus. As shown in Table 1, cAMP administration caused a small (20 per cent) increase in the wet weight of the uterus, but there was no increase in prolyl hydroxylase activity. Uterine wet weight was also unchanged from the zero control (25·3 ± 1·4 mg) at 3 (28·9 ± 2·5 mg), 4 (26·8 ± 1·2 mg), 5 (25·4 ± 1·0 mg) and 6 (28·3 ± 2·4 mg) hr after a single

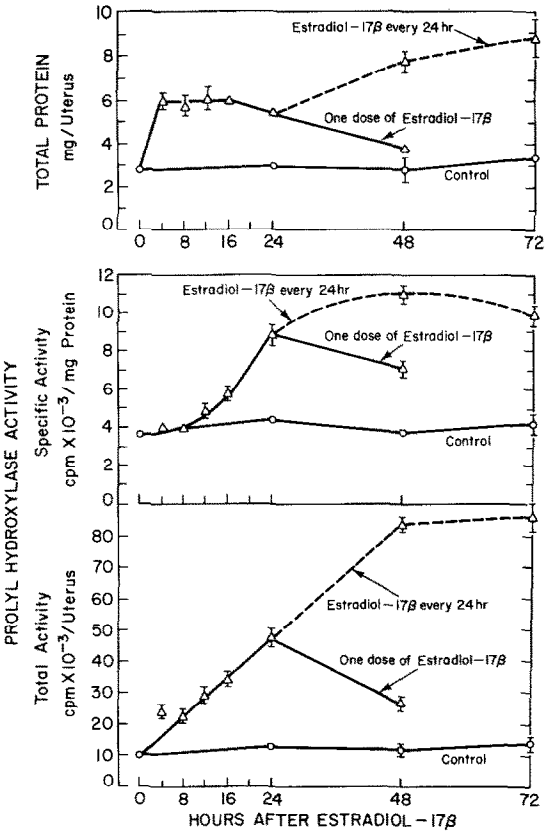

Fig. 2. Time course of the increase in prolyl hydroxylase activity in the uterus of the ovariectomized rat after the administration of estradiol-17β. Adult rats with an age variation of no more than 7 days were castrated when body weight reached 155–160 g. A single dose of estradiol-17β (5 μg) was administered intraperitoneally 22 days after castration. The controls were ovariectomized rats injected with vehicle (5% ethanol in saline). Each point is the mean value ± S.E.M. of determinations on at least four 27,000 *g* supernatants, each prepared from one to three uteri.

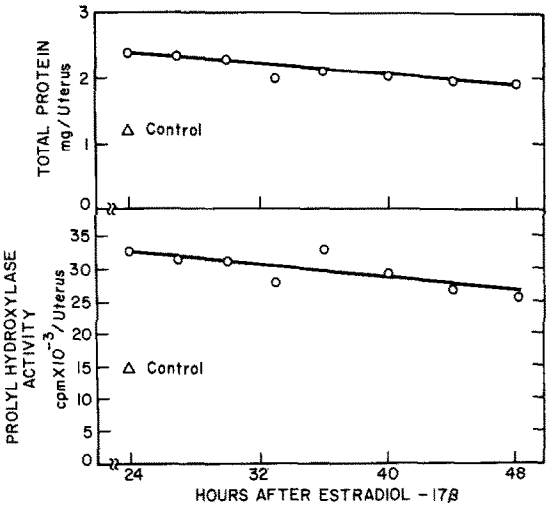

Fig. 3. Time course of the decrease in activity of prolyl hydroxylase in the uterus of the immature rat after the administration of a single dose of estradiol-17β. See legend to Fig. 1 for details.

dose of dibutyryl cAMP (5 mg, i.p.). In other groups of rats, a 5-mg dose of the latter compound was administered at zero-time and again either 2, 3 or 4 hr later. The uteri of these animals were removed and weighed at 4 (26·6 ± 0·3 mg), 6 (26·5 ± 0·5 mg) and 8 (27·6 ± 0·6 mg) hr, respectively, after the first dose. Uterine wet weight did not change with this treatment.

As shown in Table 1, theophylline caused a small (24 per cent) increase in the wet weight of the uterus, but was without effect on prolyl hydroxylase activity. Theophylline failed to augment the effect of a submaximal dose of estradiol-17β (0·01 μg) on either uterine wet weight or prolyl hydroxylase activity. The increase in uterine wet weight observed with cAMP and theophylline treatment is considerably less than that reported by other investigators [16]. It should also be noted that there was a 50 per cent mortality in theophylline-treated rats in our experiments.

Effect of inhibitors of protein and RNA synthesis on the estradiol-17β-induced increase in prolyl hydroxylase activity. Total prolyl hydroxylase activity increased approximately 2-fold in the uterus of the immature and ovariectomized rat 8 and 6 hr, respectively, after the administration of estradiol-17β (Figs. 1 and 2). This increase was inhibited in the immature rat by cycloheximide but not by actinomycin, puromycin or 5-fluorouracil, using maximally tolerable doses (Fig. 5). This dose of cycloheximide also blocked the estradiol-17β-induced increase in total protein. The estradiol-17β-induced increase in prolyl hydroxylase activity in the uterus of the ovariectomized rat was blocked about 85 per cent by cycloheximide. The increase in enzyme activity was also partially inhibited by actinomycin and 5-fluorouracil, while puromycin had no effect (Fig. 6). However, in another experiment in which the dose of puromycin was increased to 100 mg/kg, the increase in prolyl hydroxylase activity was inhibited about 90 per cent. These results suggest that the estradiol-17β-induced increase in prolyl hydroxylase activity in the uterus

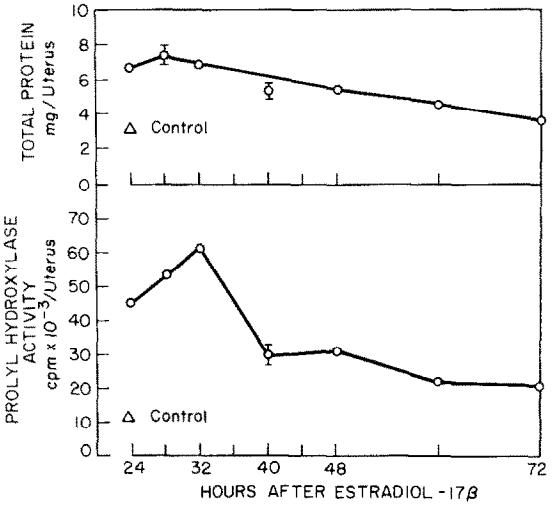

Fig. 4. Time course of the decrease in activity of prolyl hydroxylase in the uterus of the ovariectomized rat after the administration of a single dose of estradiol-17β. See legend to Fig. 2 for details.

Table 1. Effect of cAMP and theophylline on uterine wet weight and prolyl hydroxylase activity

Treatment	Wet wt of uterus (mg)	Prolyl hydroxylase	
		Specific activity (cpm × 10^{-3}/mg protein)	Total activity (cpm × 10^{-3}/uterus)
None	22·3 ± 0·7	16·7 ± 0·7	19·1 ± 0·8
cAMP*	26·7 ± 1·0†	16·8 ± 0·3	20·9 ± 1·2
None	18·9 ± 0·8	16·4 ± 1·1	15·1 ± 0·9
Estradiol-17β (0·01 μg)	26·4 ± 1·4†	20·4 ± 0·8†	26·9 ± 2·0†
Theophylline‡	23·5 ± 0·5†	13·8 ± 0·3	16·4 ± 0·4
Estradiol-17β and theophylline‡	29·2 ± 1·5	16·9 ± 0·4	23·4 ± 0·3

* A group of 21-day-old rats were given cAMP (5 mg/rat, i.p.) at 0 and 8 hr; the uteri were removed 16 hr after the first dose and weighed. The enzyme source for each assay was the 27,000 *g* supernatant from the homogenate of two pooled uteri.

† These values are significantly different from the appropriate controls ($P < 0·05$).

‡ Groups of 21-day-old rats were given a daily intraperitoneal dose of estradiol-17β (0·01 μg) for 2 consecutive days. Theophylline-treated rats were given 5 mg (i.p.) on the first day and 2·5 mg b.i.d. the second day. Theophylline was administered just prior to estradiol-17β. The appropriate vehicle was administered in the absence of other treatment. The uteri were removed from each group 24 hr after the last dose of estradiol-17β, weighed and pooled in groups of two to assay for prolyl hydroxylase. Each value is the mean activity of five assays ± S.E.M.

of the immature and ovariectomized rat is dependent, at least in part, upon protein synthesis.

Prolyl hydroxylase activity and cross-reacting protein (CRP) in the uterus of the immature and ovariectomized rat after the administration of estradiol-17β. The administration of estradiol-17β (5 μg/day) to the immature rat for 2 consecutive days resulted in a 5-fold increase in prolyl hydroxylase activity of the uterus, while the amount of CRP increased approximately 3-fold (Table 2). In contrast, prolyl hydroxylase activity and the amount of CRP decreased in the heart, kidney and lung. In the ovariectomized rat, prolyl hydroxylase activity of the uterus increased 25-fold, while CRP increased approximately 9-fold (Table 3). The prolyl hydroxylase activity did not change significantly in heart, kidney and lung. The amount of CRP increased in heart and decreased in lung.

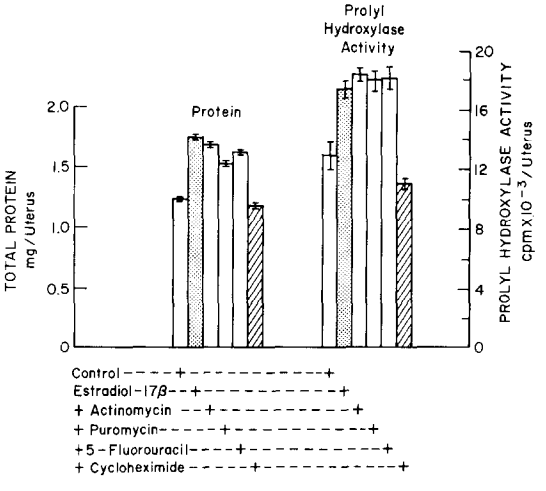

Fig. 5. Effect of actinomycin, puromycin, 5-fluorouracil and cycloheximide on the estradiol-17β-induced increase in prolyl hydroxylase activity in the uterus of the immature rat. Either 5 μg estradiol-17β or vehicle (control) was administered intraperitoneally and uteri were removed for assay of enzyme activity 8 hr later. Actinomycin (250 μg/kg), puromycin (100 mg/kg) and cycloheximide (1 mg/kg) were administered intraperitoneally 1 hr before estradiol-17β and again 4 hr later, while 5-fluorouracil (150 mg/kg) was injected only 1 hr prior to the steroid. Each value is the mean ± S.E.M. of five assays. The enzyme source for each assay was the 27,000 *g* supernatant obtained from a homogenate of two uteri.

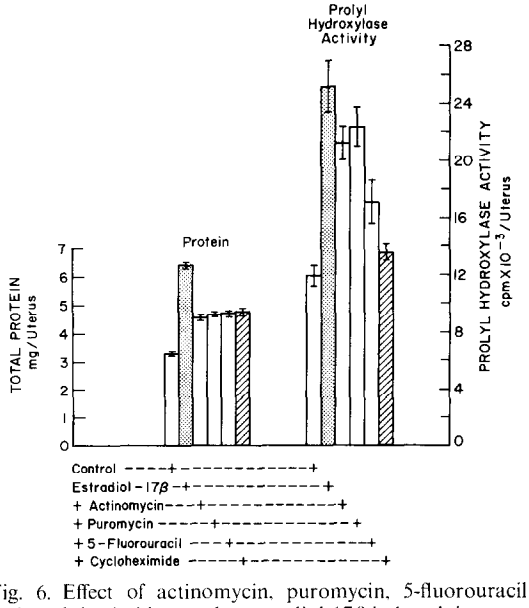

Fig. 6. Effect of actinomycin, puromycin, 5-fluorouracil and cycloheximide on the estradiol-17β-induced increase in prolyl hydroxylase activity in the uterus of the ovariectomized rat. Either 5 μg estradiol-17β or vehicle (control) was administered intraperitoneally and uteri were removed for assay of enzyme activity 6 hr later. Actinomycin (0·5 mg/kg), 5-fluorouracil (150 mg/kg) and cycloheximide (2 mg/kg) were administered intraperitoneally 1 hr before estradiol-17β, while puromycin (50 mg/kg) was injected 1 hr before and 3 hr after the steroid. Each value is the mean ± S.E.M. of five assays. The enzyme source for each assay was the 27,000 *g* supernatant obtained from a homogenate of two uteri.

Table 2. Prolyl hydroxylase and cross-reacting protein in different tissues of the immature female rat after administration of estradiol-17β*

Tissue	Control (cpm × 10^{-4}/organ)		Estradiol-17β (cpm × 10^{-4}/organ)	
	Prolyl hydroxylase activity	Cross-reacting protein	Prolyl hydroxylase activity	Cross-reacting protein
Uterus	3·6 ± 0·1	59·4 ± 1·6	17·9 ± 0·3†	194·1 ± 0·6†
Heart	12·4 ± 0·2	110·4 ± 3·5	9·8 ± 0·4†	87·2 ± 2·0†
Kidney	24·8 ± 0·1	332·3 ± 7·2	15·0 ± 1·4†	201·0 ± 7·9†
Lung	74·4 ± 1·2	974·6 ± 29·8	63·7 ± 4·4†	686·3 ± 19·6†

* Estradiol-17β (5 μg/day, i.p.) was administered for 2 consecutive days; tissues were removed 24 hr after the second dose. The tissues were homogenized in 0·01 M Tris–HCl buffer (pH 7·4) containing 0·25 M sucrose, 0·1% Triton X-100, 10^{-4} M dithiothreitol and 10^{-5} M EDTA. The 27,000 *g* supernatants were assayed for prolyl hydroxylase activity and cross-reacting protein as described in Materials and Methods. The results are expressed as the mean ± standard error (n = 6).

† These values are significantly different from the controls ($P < 0·05$).

DISCUSSION

The hydroxylation of proline has been recognized as a key reaction in the biosynthesis of collagen, even though the function of hydroxyproline was not understood [4–8]. It is now known that the thermal stability of the triple helical structure of the collagen molecule is a function of the hydroxyproline content of the molecule [2, 3]. Moreover, the evidence indicates that the triple helical configuration is required for the normal secretion of collagen into the extracellular space [3, 18]. These findings demonstrate the importance of prolyl hydroxylase in collagen biosynthesis.

The estradiol-17β-stimulated uterus of the rat provides a convenient model *in vivo* for the study of the regulation of collagen biosynthesis. The purpose of these investigations was to determine whether the increase in prolyl hydroxylase activity in the uterus after the administration of estradiol-17β was due to *de novo* synthesis of enzyme protein. Because of the toxicity of inhibitors of protein and RNA synthesis, inhibition of the estradiol-17β-induced increase in prolyl hydroxylase activity was only attempted for the initial 6–8 hr. In the immature rat, this effect of estradiol-17β was inhibited 85 per cent by cycloheximide, while this response was unaffected by maximally tolerated doses of actinomycin, puromycin and 5-fluorouracil. These inhibitors were partially effective in blocking this response in the ovariectomized rat. This was probably related to the ability of the adult animal to tolerate higher doses of these drugs. These results suggest that the stimulatory effect of estradiol-17β on prolyl hydroxylase activity is related in part to *de novo* synthesis of enzyme protein.

Prolyl hydroxylase is present in all mammalian tissues tested, in an inactive as well as an active form [14]. This has been demonstrated by use of a specific enzyme immunoassay [14, 19]. This procedure was used to quantitate total prolyl hydroxylase antigen, active prolyl hydroxylase and cross-reacting protein. After estradiol-17β administration, there is a marked increase in the amount of this antigen in the uterus of both the immature and ovariectomized rat. These findings provide further evidence that the increase in prolyl hydroxylase activity is accompanied by *de novo* synthesis of enzyme protein. However, it is possible that a decrease in the degradation of enzyme protein or other post-transcriptional effects of estradiol-17β could also account for the increase in the activity of

Table 3. Prolyl hydroxylase and cross-reacting protein in different tissues of the ovariectomized rat after administration of estradiol-17β*

Tissue	Control (cpm × 10^{-4}/organ)		Estradiol-17β (cpm × 10^{-4}/organ)	
	Prolyl hydroxylase activity	Cross-reacting protein	Prolyl hydroxylase activity	Cross-reacting protein
Uterus	1·1 ± 0·2	28·5 ± 2·2	27·9 ± 1·5†	273·4 ± 5·2†
Heart	18·9 ± 3·6	146·5 ± 31·1	25·2 ± 1·2	234·4 ± 1·5†
Kidney	49·2 ± 5·8	482·7 ± 2·8	42·1 ± 6·5	449·0 ± 40·4
Lung	137·0 ± 13·5	893·0 ± 62·3	110·0 ± 7·1	599·4 ± 32·5†

* See legend of Table 2 for experimental details. The results are expressed as the mean ± standard error (n = 4).

† These values are significantly different from the controls ($P < 0·05$).

prolyl hydroxylase. Earlier results in L-929 fibroblasts suggested that inactive enzyme protein can be converted to active enzyme [20, 21]. The decrease in the ratio of inactive to active enzyme in the uterus after estradiol-17β could be due to a similar conversion. It is possible that this mechanism, is in part, responsible for the increase in prolyl hydroxylase activity seen after the administration of estradiol-17β.

In addition to altering prolyl hydroxylase activity in the uterus, estradiol-17β administration also caused significant changes in the quantity of this enzyme in heart, lung and kidney of the immature rat. In contrast to findings in the uterus, both the prolyl hydroxylase activity and the amount of cross-reacting protein were reduced in these tissues.

Estradiol-17β causes an increase in the activity of other enzymes in the uterus such as phosphofructokinase [22]. It has also been reported that cAMP and dibutyryl cAMP cause a marked increase in uterine wet weight and in the activity of uterine phosphofructokinase [16, 17]. These investigators suggest that the estradiol-17β-stimulated increases are mediated through cyclic nucleotides. In our studies, a small increase in uterine wet weight occurred after the administration of large intraperitoneal doses of cAMP but not after dibutyryl cAMP; the activity of prolyl hydroxylase was unaffected. Theophylline has been reported to significantly increase uterine wet weight (50 per cent) and the activity of hexokinase, phosphofructokinase and pyruvate kinase in the immature rat [23]. In the latter study, theophylline was also reported to potentiate the action of a submaximal dose of estradiol-17β. A more modest increase in uterine wet weight (24 per cent) was observed in our studies but there was no augmentation of the effect of a submaximal dose of estradiol-17β. Although the dose of theophylline we used was less than that reported above as stimulating these effects, a 50 per cent mortality occurred. Theophylline administration did not affect prolyl hydroxylase activity of the uterus. The reason for these differences is not immediately apparent but could be related to experimental differences such as the route of theophylline administration or the strain of rat employed. There are other reports, however, whose results are similar to our own [24, 25]. These findings do not support the suggestion that cyclic AMP mediates the action of estrogens in the uterus.

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