Oestradiol Inhibits Collagen Breakdown in the Involuting Rat Uterus

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1. The earlier observation (Woessner, 1969) of oestradiol inhibition of collagen breakdown is confirmed and extended. Administration of 100 μ g of oestradiol-17 β /day to parturient rats strongly inhibits the loss of collagen from the involuting uterus. Three experiments show that this effect is due to an inhibition of collagen degradation rather than to a stimulation of collagen synthesis. 2. Uterine collagen was labelled with hydroxy[14 C]-proline by the administration of [14 C]proline near the end of pregnancy. By 3 days *post partum*, control uteri lost 83% of their collagen and 90% of their hydroxy[14 C]proline. Uteri from oestradiol-treated rats lost only 50% of both total and labelled hydroxyproline, with no decrease in the specific radioactivity of the hydroxyproline. 3. Incorporation of [14 C]proline into uterine collagen hydroxyproline *in vivo* was not affected by oestradiol treatment. 4. Urinary excretion of hydroxyproline was increased in post-partum control rats and decreased in oestradiol-treated rats. 5. An enzyme capable of cleaving 4-phenylazobenzoyloxycarbonyl-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine (a substrate for clostridial collagenase) increased in activity in the post-partum uterus and was unaffected by oestradiol treatment. 6. Uterine homogenates digested uterine collagen extensively at pH 3.2. This digestion was unaffected by the oestradiol treatment. 7. Lysosomal fractions prepared by density-gradient centrifugation of uterine homogenates contained coincident peaks of cathepsin D activity and peptide-bound hydroxyproline. The cathepsin D and hydroxyproline contents of this peak were unaffected by oestradiol treatment.

Involution of the rat uterus during the post-partum period is an extensive remodelling process accompanied by the rapid removal of collagen; as much as 85% of the total collagen present in the uterus at the end of pregnancy is removed within 4 days (Harkness & Moralee, 1956; Woessner, 1962). This loss of collagen is dramatically decreased by the administration of oestradiol-17 β at the time of parturition (Woessner, 1969). The fact that the uteri from oestrogen-treated rats contain much more collagen than do uteri from control rats by 3 days *post partum* might be due either to an inhibition of collagen breakdown or to the stimulation of new collagen synthesis. The oestrogenic hormones generally display anabolic rather than anticatabolic activities. However, the amount of free hydroxyproline in the involuting uterus was markedly decreased by oestradiol treatment, and this was taken to be good evidence for an inhibition of collagen degradation (Woessner, 1969). Other evidence for an inhibitory effect of oestrogens on collagen breakdown in rat skin and bones has been presented by Skosey &

Kappas (1967) and Orimo *et al.* (1971). However, we considered that the finding of an anticatabolic effect of oestradiol on collagen metabolism might be placed on a firmer basis by the use of isotopic labelling. This seemed especially important in view of reports (Henneman, 1968, 1971) suggesting that oestradiol stimulates both synthesis and degradation of collagen. In the present paper we adduce evidence from the study of the breakdown of labelled collagen, the synthesis of labelled collagen and the appearance of collagen catabolites in urine, which points to an anticatabolic action of oestradiol on collagen resorption.

The physiological mechanisms by which collagen is degraded during uterine involution have not been fully elucidated. Nor has it been possible, thus far, to show at what point in these mechanisms oestradiol exerts its effect. It was shown earlier that lysosomal hydrolase activities increased during involution (Woessner, 1965) and that lysosomal cathepsins could attack uterine collagen at acid pH values (Woessner, 1962). More recently, a number of workers (Brandes & Anton, 1969*a,b*; Parakkal, 1969; Schwarz & Guldner, 1967; Dessouky, 1971) have provided electron-microscopic evidence that uterine

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collagen is extensively phagocytized by macrophages and is brought into digestive vacuoles containing lysosomal hydrolases.

On the other hand, collagenase activity has been detected in cultures of involuting uterus (Jeffrey & Gross, 1970). This collagenase is capable of degrading collagen fibres at neutral pH values; it would presumably act outside the cell rather than inside digestive vacuoles. Although attempts to demonstrate collagenase activity directly in uterine homogenates have met with little success (Schaub, 1963; Morrione & Seifter, 1962; Woessner & Brewer, 1963; Jeffrey & Gross, 1970), we have recently shown that the enzyme is present and is presumably bound firmly to the collagen fibres (Ryan & Woessner, 1971a). A second enzyme, which cleaves a pentapeptide used for the assay of bacterial collagenase, has been found in the involuting rat uterus by Strauch (1970). This enzyme is sometimes designated as collagenase, although there is no evidence that it digests collagen.

In the present study we have looked for effects of oestradiol on the lysosomal system of the rat uterus and on the enzyme that cleaves the pentapeptide (PZ-peptidase).

Materials and Methods

Treatment of animals

Female white rats at 17 days of gestation were purchased from Sprague-Dawley, Inc., Madison, Wis., U.S.A. They were kept in individual cages and fed on Purina Rat Chow and given water *ad lib*. The time of delivery was recorded and the young were left in the cage to permit the mother to nurse. Rats which failed to nurse or to deliver live young, or which were later found to have retained placentae or foetuses, were discarded. Animals receiving oestradiol-17 β [oestra-1,3,5(10)-triene-3,17 β -diol; Mann Research Laboratories, New York, N.Y., U.S.A.] were injected intraperitoneally with 0.10 ml of a suspension of the hormone (100 μ g) in 0.9% NaCl shortly after delivery was complete and on each successive day until death. Controls received injections of 0.9% NaCl only. Rats were first stunned with a blow, then killed by cervical dislocation. Uteri were dissected free of fatty tissue and placental sites, washed in cold 0.9% NaCl, blotted and weighed.

Studies of collagen metabolism

To measure the resorption of prelabelled collagen, L-[U-¹⁴C]proline (New England Nuclear Corp., Boston, Mass., U.S.A.; 209 mCi/mmol) was dissolved in 0.9% NaCl and adjusted to pH 7 with a final radioactivity of 50 μ Ci/ml. Rats were given a series of three intraperitoneal injections over a 24 h period on the estimated days 18 and 19 of gestation for a total dose of approx. 3 μ Ci/100 g body wt. Several

injections over a period of time were used in an attempt to minimize individual variations in the incorporation of label. For the studies of collagen synthesis, rats were given a single intraperitoneal dose of 3 or 12 μ Ci of [¹⁴C]proline 3 h before removal of the uteri. These studies were done on day 19 of gestation and the second day after parturition. Incorporation of label was found to be proportional to the administered dose, so the results are presented as d.p.m./ μ Ci of [¹⁴C]proline injected.

In both types of metabolic study the uterine tissue was minced, homogenized in 5 vol. of cold water, then dialysed exhaustively against water in the cold. The homogenates were then hydrolysed in 6M-HCl and analysed for total hydroxyproline (Woessner, 1961) and for hydroxy[¹⁴C]proline (Juva & Prockop, 1966). Samples were counted for radioactivity in a Packard Tri-Carb model 574 liquid-scintillation spectrometer (machine efficiency 75–78%) and then corrected for background and to 100% efficiency by the use of external standards. The scintillation fluid contained 80 g of naphthalene, 7 g of 2,5-diphenyloxazole and 300 mg of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in 1 litre of toluene-dioxan-ethanol (4:4:2.4, by vol.). The incorporation of label into non-collagenous protein was calculated from the total radioactivity (d.p.m.) of the hydrolysate by subtracting the contribution from collagen, assuming a proline/hydroxyproline ratio of 1:1.11 for rat uterine collagen (Kao *et al.*, 1968).

Rat urine was collected continuously in metabolic cages, and 24 h samples were chromatographed on Dowex 50W (X2) resin by the method of Koevoet & Baars (1969), hydrolysed in HCl and assayed for hydroxyproline (Woessner, 1961).

Studies with lysosome

Uterine tissue was homogenized in 10 vol. of 0.25M-sucrose containing 1mM-EDTA and 0.01M-sodium phosphate, pH 7.4. Samples were layered on 35–65% (w/v) sucrose density gradients and centrifuged in the SW25.1 rotor of the Beckman model L ultracentrifuge at 60000g (r_{av} . 9.1 cm) for 16 h to reach equilibrium. Fractions (1 ml) were collected from the bottom of the tube, and samples were assayed for cathepsin D (Woessner & Brewer, 1963). The fractions were dialysed to remove sucrose, hydrolysed in HCl and assayed for hydroxyproline (Woessner, 1961, method II).

Digestion of collagen by uterine cathepsins

Tissue was homogenized in 10 vol. of 0.9% NaCl, the pH was adjusted to 3.2 and 2.5 vol. of 0.5M-sodium acetate buffer, pH 3.2, was added. Samples were incubated overnight at 37°C and 0°C with shaking. They were then centrifuged at 30000g (r_{av} .

5.9 cm) for 30 min. The supernatants and pellets were carefully separated, hydrolysed and assayed for hydroxyproline. The digestion of the endogenous uterine collagen by the uterine cathepsins was determined from the amount of hydroxyproline that had been transferred from the pellet to the supernatant after incubation at 37°C.

Assay of PZ-peptidase

Tissue was homogenized in 2 mM-calcium acetate and centrifuged at low speed (600g). The supernatant was tested for its ability to cleave 4-phenylazobenzoyloxycarbonyl-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine (PZ-peptide; Mann Research Laboratories) by using the assay developed by Wunsch & Heidrich (1963). High-voltage paper electrophoresis and amino acid chromatography were used to establish that uterine extracts cleaved the Leu-Gly bond.

Results

Metabolism of collagen during involution

We studied the effect of oestradiol on collagen metabolism during uterine involution by examining three parameters: collagen resorption, collagen synthesis and the excretion of hydroxyproline in the urine. To measure the extent of collagen resorption, uterine collagen was labelled *in vivo* with [^{14}C]proline during the final 3–4 days of gestation, and the animals were randomly assigned to three groups. The amount of total hydroxyproline and of hydroxy-

[^{14}C]proline present in the uterus at parturition was determined for group 1 (Table 1). The loss of total and labelled hydroxyproline by the third day *post partum* was measured in control (group 2) and oestrogen-treated rats (group 3). In agreement with previous results (Woessner, 1965, 1969), approx. 80% of the total collagen present at the time of parturition is lost within 3 days during normal involution. When rats are injected with 100 μg of oestrogen daily, this loss is only 50%. Similar results are observed in the loss of labelled collagen, i.e. that material present at the end of pregnancy (approx. 90% of the hydroxy[^{14}C]proline) is removed in the control group whereas only 48% is lost during treatment with oestrogen. Thus the oestrogen-mediated inhibition of resorption of prelabelled collagen exactly matches the inhibition of resorption of total collagen. The specific radioactivity of the collagen from the uteri of rats treated with oestrogen for 3 days is unchanged from the value at parturition. In contrast to the resorption of collagen, the resorption of prelabelled non-collagenous protein is not affected by treatment with oestrogen.

The rate of synthesis of uterine collagen was compared in the two groups by measuring the incorporation of [^{14}C]proline *in vivo* (Table 2). Rats were injected with label at noon of the second day *post partum* and were killed 3 h later. The incorporation of label into uterine collagen (hydroxyproline) during the late pre-partum period (day 19) was also determined to compare collagen synthesis during the post-partum period with that occurring at a time of

Table 1. *Effect of oestradiol on collagen resorption during uterine involution*

Figures in parentheses are the number of rats in each group. Values are presented as means $\pm$ s.d. Significant differences from control: * $P < 0.001$ and ** $P < 0.05$.

	Parturition: day 0 (14)	Control: day 3 (16)	Oestradiol-treated: day 3 (14)
Wt. of rat (g)	280 $\pm$ 30	300 $\pm$ 40	275 $\pm$ 35
Wt. of uterus (mg)	2740 $\pm$ 460	620 $\pm$ 70	1000 $\pm$ 145*
Content of hydroxyproline (μg /uterus)	8660 $\pm$ 1160	1500 $\pm$ 460	4300 $\pm$ 670*
Radioactivity of hydroxyproline (d.p.m./uterus)†	32500 $\pm$ 1970	3300 $\pm$ 2300	16900 $\pm$ 9700*
Sp. radioactivity of hydroxyproline (d.p.m./ μg of hydroxyproline)	3.90 $\pm$ 2.50	2.30 $\pm$ 1.40	3.85 $\pm$ 1.95**
Radioactivity of non-collagen protein (d.p.m./uterus)	182900 $\pm$ 87300	24800 $\pm$ 15500	33500 $\pm$ 11300
Percentage of value at parturition			
Hydroxyproline (μg /uterus)		17	50
Hydroxyproline (d.p.m./uterus)		10	52
Non-collagen protein (d.p.m./uterus)		14	18

† Label was introduced on days 18 and 19 of pregnancy.

Table 2. *Effect of oestradiol on collagen synthesis during uterine involution*

[¹⁴C]Proline was injected 3h before the animals were killed. Values are presented as means $\pm$ S.D.; the number of animals is given in parentheses. Significant differences from control: * $P < 0.001$ and ** $P < 0.05$.

	Control: day 2 (5)	Oestradiol-treated: day 2 (7)	Pre-partum (7)
Wt. of rat (g)	307 $\pm$ 14	265 $\pm$ 10*	
Wt. of uterus (mg)	885 $\pm$ 150	1065 $\pm$ 90**	3120 $\pm$ 380*
Content of hydroxyproline (μ g/uterus)	3450 $\pm$ 1050	4610 $\pm$ 670**	8970 $\pm$ 2140*
Radioactivity of hydroxyproline (d.p.m./g of uterus)	500 $\pm$ 210	640 $\pm$ 170	1600 $\pm$ 1110**
Sp. radioactivity of hydroxyproline (d.p.m./ μ g of hydroxyproline)	0.143 $\pm$ 0.091	0.147 $\pm$ 0.032	0.560 $\pm$ 0.379*
Radioactivity of non-collagen protein (d.p.m./g of uterus)	4613 $\pm$ 1528	5903 $\pm$ 1109	10984 $\pm$ 3273*

rapid growth and synthesis. The synthesis of both collagen and non-collagen protein appeared to be slow at 2 days *post partum* compared with the pre-partum synthesis rate. There was no significant change in the incorporation of labelled proline into uterine collagen after oestrogen administration.

The excretion of hydroxyproline in the urine was determined during involution (Table 3). By the second day *post partum*, the amount of hydroxyproline excreted within a 24h period is significantly elevated above the average value observed during the final 3 days of pregnancy. This increase in hydroxyproline in the urine occurs at the time that collagen is being most rapidly lost from the uterus (Woessner, 1965). Oestrogen treatment results in a very significant decrease in hydroxyproline in the urine relative to that observed during normal involution as well as during the pre-partum period.

Uterine lysosomes

The total activity of several uterine lysosomal hydrolases such as cathepsin D, acid phosphatase and β -glucuronidase was essentially unchanged by treatment with oestradiol during involution (Woessner, 1965). Nevertheless, the possibility still remained that oestrogen might inhibit collagen degradation by altering lysosomal stability or by interfering with phagocytosis or some step in intralysosomal digestion. We prepared lysosome-rich fractions from uteri of oestrogen-treated and untreated rats to compare the stability of lysosomes prepared from both tissues. The recovery of intact lysosomes from both sources was approx. 20%; no increase in the susceptibility of lysosomes to mechanical disruption was apparent after oestrogen treatment. Further, oestrogen did not increase lysosomal fragility as

studied by incubation at 37°C at pH 7.4 in iso-osmotic sucrose. Approx. 17% of the total cathepsin D present in isolated lysosomes prepared from uteri of control rats was released after incubation for 40min; this proportion was not altered by oestradiol treatment.

Lysosome-rich fractions were then centrifuged to equilibrium in 35–65% linear sucrose density gradients. The individual fractions were assayed for cathepsin D and a sharp peak of activity was consistently observed at a density of approx. 1.18g/ml. A typical separation is illustrated in Fig. 1. After dialysis and hydrolysis of pooled fractions, hydroxyproline was consistently found to be associated with this peak. Treatment of the lysosome-rich fraction with Triton X-100 before gradient centrifugation prevented most of the cathepsin D and the non-diffusible hydroxyproline-containing material from entering the gradient (Fig. 1). Lysosomes were prepared from uteri removed from oestrogen-treated rats on the second or third day of involution and compared with those from untreated animals; no differences were found in the equilibrium density of the peak or in the ratio of hydroxyproline to cathepsin D in this peak.

It has also been shown that uterine collagen could be digested by endogenous cathepsins at acid pH values (Woessner, 1962). These studies were repeated with tissue from control and oestradiol-treated rats (Fig. 2). In controls, 90% of the collagen is digested in overnight incubation; this percentage decreases as involution progresses. In tissue from the oestradiol-treated rats, the digestion of collagen remains at 90% throughout the 5-day period. Thus the digestive capacity of the uterus at acid pH values is not impaired by oestrogen treatment, nor is the collagen rendered less susceptible to digestion.

Table 3. *Hydroxyproline excretion in the urine*

Results are presented as means $\pm$ s.d. Column (A) lists values of *P* for results significantly different from the pre-partum value and column (B) lists values of *P* for results significantly different from the control values after treatment with oestradiol (Student's *t* test).

Group	No. of animals	Hydroxyproline excreted ($\mu\text{g}/24\text{h}$)	<i>P</i>	
			(A)	(B)
Pre-partum	22*	740 $\pm$ 210		
Control				
Day 1	14	880 $\pm$ 200	0.01	
Day 2	13	1130 $\pm$ 350		
Day 3	9	1090 $\pm$ 460		
Oestradiol-treated				
Day 1	10	470 $\pm$ 200	0.005	0.001
Day 2	10	510 $\pm$ 240	0.01	0.001
Day 3	8	510 $\pm$ 260	0.05	0.01

* Seven animals were studied daily during the last 3–4 days of gestation. The daily excretion remained constant during this period.

Fig. 1. *Localization of hydroxyproline and cathepsin D activity after centrifugation of homogenates of uterus for 3 h at 60000g through a 35–65% sucrose density gradient*

For experimental details see the text. Fraction volume is 1 ml. (a) Pattern of homogenate of 3-day post-partum uterus; (b) pattern of the same homogenate treated with Triton X-100 before centrifugation., Concn. of sucrose; —, hydroxyproline content; ●, cathepsin D activity.

PZ-peptidase activity

The activity of a peptidase capable of cleaving the pentapeptide substrate for bacterial collagenase was determined in homogenates of the involuting uterus (Fig. 3). The total activity per uterus was highest immediately after parturition and then rapidly decreased. Oestrogen treatment did not affect either the total peptidase activity or the decrease in activity that occurred during involution. However, since the uteri from the treated animals remained larger than those from the controls, the concentration of PZ-peptidase activity was considerably lower than in the uteri of untreated rats by the second day *post partum*.

Discussion

Inhibition of collagen degradation by oestradiol-17 β

The original observation (Woessner, 1969) was that at 3 days *post partum* uteri from rats that had received oestrogen from the time of parturition contained significantly more collagen than did uteri from control animals. This observation is subject to two possible interpretations. The first is that oestrogen inhibits collagen degradation during this period; the second is that degradation is unaffected whereas the synthesis of new collagen is increased to replace part of that lost by degradation. On the basis of the decrease in free hydroxyproline in uteri from oestrogen-treated animals, it appeared that the main effect of oestrogen was to block the breakdown of collagen. The results presented in the present paper are consistent with this hypothesis.

Fig. 2. Digestion of collagen at acid pH values in homogenates of uteri

For experimental details see the Materials and Methods section. o, Control rats; ●, oestradiol-treated rats. The pre-partum value was obtained approximately 1 day before parturition.

Fig. 3. PZ-peptidase activity at pH7.1 in uteri

For experimental details see the text. o, Control rats; ●, oestradiol-treated rats; each point represents three animals.

Oestrogen treatment partially blocks the loss of label from uterine collagen during involution (Table 1). By 3 days *post partum* uteri from oestrogen-treated rats contain 50% of the labelled hydroxyproline present at the time of parturition; in contrast to this, untreated animals retain only 10%. Further, the loss of hydroxyproline labelled before parturition exactly parallels the net loss of total hydroxyproline. These results agree well with the postulate that the degradation of collagen is decreased by oestrogen treatment. They do not agree with the hypothesis that oestrogen stimulates new collagen synthesis since there is no decrease in the specific radioactivity of the collagen and since only 50% of the label is lost as compared with 90% lost from the controls.

The incorporation of label during the post-partum period into previously unlabelled collagen is not stimulated by oestrogen treatment (Table 2). It might be argued that the oestrogen-treated animals had a larger metabolic pool of free proline, which diluted the added label and masked an increase in collagen synthesis. Although this possibility cannot be excluded, it does not seem likely. A considerable part of the uterine proline pool is presumed to arise from

the extensive breakdown of uterine protein. Table 1 indicated that non-collagen protein was breaking down at control rates, or slightly slower in the oestrogen-treated animals, whereas collagen was breaking down much more slowly. Thus these two sources would, if anything, provide decreased proline pools in oestrogen-treated animals. The finding of a lack of stimulation of collagen synthesis by oestradiol is consistent with the failure to observe any dilution of the specific radioactivity of collagen during involution in the oestrogen-treated rat (Table 1).

The amount of hydroxyproline excreted in the urine was used as an indicator of collagen metabolism throughout the body. An increase in the excretion of hydroxyproline could be due to an increase in either collagen degradation or collagen synthesis, since some of the newly synthesized forms of collagen are particularly susceptible to degradation and turn over rapidly (Nimni *et al.*, 1967). Conversely, decreased amounts of hydroxyproline in the urine would indicate decreased collagen metabolism (Aer, 1968; Kohler & Pechet, 1966). The decrease in the excretion of hydroxyproline shown in Table 2 supports the contention that oestrogen decreases the degradation of collagen. It is not known how much of the hydroxy-

proline in the urine derives from the uterus. If we use the pre-partum value as a control, then of the 7000 μg of uterine hydroxyproline lost in the first 3 days, only 900 μg (13%) would be accounted for by urinary excretion. This seems a reasonable value (cf. Kivirikko, 1970); however, if we accept this, then the oestrogen effect on hydroxyproline in the urine seems extremely large in proportion to the inhibition of collagen breakdown in the uterus. Even if the entire urinary output of the control were derived from the uterus, the oestrogen effect would still be too large. It must be concluded therefore that the hydroxyproline in the urine is derived from other sources in addition to the uterus, and that oestrogen inhibits degradation of collagen in these sources as well. Alternatively, oestrogen might modify the transport of hydroxyproline peptides through the kidney.

There have been conflicting reports on the urinary excretion of hydroxyproline in the post-partum period in women. Klein (1964) studied three patients and found a maximum excretion of hydroxyproline at 2–6 days *post partum*. This would agree with the present study and the known rate of involution of the human uterus (Woessner & Brewer, 1963). Klein & Yen (1970) found that only two of six patients showed a peak in excretion of hydroxyproline in the post-partum period whereas the other four reached a peak before parturition. Unfortunately, their study presents almost no results for the period of greatest interest, between 2 and 14 days *post partum*. However, there is no question that the concentration of hydroxyproline in urine is well above normal values during post-partum involution. Klein & Yen (1970) further argue that since the urinary amounts do not account for more than 10% of the uterine hydroxyproline, and since the uterus is low in the prolidase activity required to cleave the hydroxyproline peptides that would otherwise appear in the urine, the uterine collagen must therefore be removed by a non-degradative process that preserves the collagen molecule more or less intact.

This argument is based on a faulty interpretation of the work of Smith (1948), who in fact showed considerable amounts of prolidase in the human uterus. The argument also ignores the high prolidase activity and concentrations of free hydroxyproline found in the rat uterus (Woessner, 1962). We believe that most of the collagen is degraded to the level of small peptides and free amino acids and that the free hydroxyproline undergoes further metabolism (Wolf & Berger, 1958) so that only a small proportion of the breakdown products of collagen is found in the urine.

The observations that oestradiol decreases the loss of labelled uterine collagen, does not alter the incorporation of [^{14}C]proline into uterine collagen and decreases free hydroxyproline in the uterus and total hydroxyproline in the urine, all lead to the inescapable

conclusion that oestradiol inhibits collagen degradation in the uterus and probably other tissues as well. Several lines of evidence support the contention that oestrogen, at least in pharmacological doses, inhibits collagen breakdown in a variety of tissues. At least four studies show that treatment of adult women with oestrogen leads to decreased urinary excretion of hydroxyproline (Young & Nordin, 1967; Young *et al.*, 1968; Katz & Kappas, 1968; Schwartz *et al.*, 1969). Oestrogen has a similar effect in the treatment of osteoporotic women (Riggs *et al.*, 1969), and the results of Orimo *et al.* (1971) show that in rats there is a direct effect on the loss of collagen from bone. Ebling & Hale (1966) show that the hydroxyproline content of rat skin varies with the hair cycle and that oestrogen decreases the fall in the collagen content. Other examples of oestrogen inhibition of collagen breakdown were cited by Woessner (1969).

Henneman (1968, 1971) has suggested that oestradiol stimulates collagen synthesis and breakdown in various guinea-pig tissues. Guinea pigs were given [^{14}C]proline over long periods of time to label the collagen; then oestrogen was administered for 5 weeks. This caused the uterus to increase in weight, collagen content and [^{14}C]hydroxyproline content compared with controls. The interpretation (Henneman, 1971) is that oestrogen stimulated the degradation of labelled collagen from other tissue sources and stimulated the synthesis of new collagen from these degradation products. Such an explanation appears unlikely. The uterus collagen has one of the shortest half-lives known (Kao *et al.*, 1961). It is difficult to see how other tissues could contribute precursors of higher specific radioactivity than those already in the uterus. On the other hand, if oestradiol decreases degradation, then it is easy to explain why the treated uteri would contain more collagen at the end of 5 weeks and why they would then retain more of their initial radioactivity than the controls at the end of this time. Similar arguments could be advanced for the findings on guinea-pig bone (Henneman, 1971). In any dynamic system of collagen metabolism it is essential to evaluate separately the relative contributions of synthesis and degradation to the net changes in collagen content.

Role of lysosomes and of PZ-peptidase

We consistently found non-diffusible hydroxyproline associated with the peak of cathepsin D activity after isopycnic density-gradient centrifugation (Fig. 1a). If the homogenates were treated with Triton X-100, both the hydroxyproline and cathepsin D remained in the supernatant and no longer moved on centrifugation to the original lysosome position (Fig. 1b). These results are intriguing and suggest that the hydroxyproline may be associated with

membranous structures such as the lysosomes. These biochemical observations may correspond to the electron-microscopic picture of the involuting uterus, which shows collagen fragments within lysosome-like structures in macrophages (see the introduction).

The function of lysosomes in degradation of collagen appears to be unimpaired by treatment with oestrogen. No differences were detected in the ratio of cathepsin D to hydroxyproline in the lysosomal peak obtained from uteri of control or treated rats; this suggests that the ability of uterine macrophages to phagocytize collagen is unaffected. Further, collagen from the uteri of oestrogen-treated rats is readily digested by the tissue acid proteinases (Fig. 2). These results, together with the previous failure to find significant alterations in the activities of the lysosomal hydrolases (Woessner, 1969), would seem to eliminate the lysosomal digestive system as a mediator of the oestrogen effects.

The behaviour of the PZ-peptidase in normal involution (Fig. 3) is similar to that reported by Strauch (1970). The uterine content shows an increase in the post-partum period and the activity per g of tissue reaches a peak at 2–3 days. Platt & Frackenpohl (1971) also showed an increase in this enzyme in the post-partum rat uterus, but they failed to specify whether they measured total uterine enzyme activity or enzyme activity per g of tissue. We conclude that this enzyme is not significantly affected by oestrogen treatment. Its role in collagen breakdown remains to be determined; Strauch (1970) calls it a collagenase. However, we have not been able to digest collagen with preparations of this enzyme, and we have been able to separate it from a uterine enzyme that does digest collagen (J. N. Ryan and J. F. Woessner, unpublished work). Harper & Gross (1970) have obtained similar results for the collagenase and peptidase activities from cultures of tadpole tail fin.

In conclusion, we have found that oestradiol inhibits collagen breakdown in the involuting uterus, and it does not appear to act on several enzyme systems postulated or believed to be implicated in collagen catabolism. The most likely remaining mechanism by which oestrogen could inhibit degradation of collagen would be through an effect on the activity of uterine collagenase. We have found that uterine collagenase activity increases during the time of most rapid collagen loss and that this activity is decreased by treatment with oestrogen (Ryan & Woessner, 1971b). However, the true explanation is probably more complicated than this finding would suggest, since uterine tissue in culture responds to added oestradiol by the production of more collagenase (Jeffrey *et al.*, 1971).

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