Inhibition by Oestrogen of Collagen Breakdown in the Involuting Rat Uterus

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(Received 29 October 1968)

1. Both the post-partum involution of the rat uterus and the rapid breakdown of collagen that accompanies it are extensively inhibited by oestrogenic hormones. In the normal rat, 85% of the uterine collagen is degraded within 4 days after parturition; in rats treated with 100 μ g. of 17 β -oestradiol/day, only 35% of uterine collagen is broken down in the same period. 2. Similar effects are produced by diethylstilboestrol if the dose is increased tenfold. 3. Collagen breakdown is inhibited to a greater extent than is the loss of wet weight by oestradiol but not by diethylstilboestrol. 4. The oestrogens appear to act by blocking the breakdown of collagen. There is a greatly decreased concentration of free hydroxyproline in the uterus of treated animals. 5. Acid hydrolase concentrations (β -glucuronidase, β -galactosidase, cathepsin D and acid phosphatase) in the uterus are decreased by oestrogen treatment compared with controls, but the total amounts of these enzymes in the uterus are somewhat elevated. Oestrogens do not appear to inhibit collagen breakdown by altering the concentration and total amount of acid hydrolases.

The involuting rat uterus provides a striking model system for the study of collagen breakdown processes. During the resorption of uterine tissue that follows parturition, there is an extremely rapid loss of collagen. About 85% of the collagen is broken down within a 4-day period (Harkness & Moralee, 1956). An important consequence of this finding is that experimental treatments that alter the rate of this breakdown process should be readily detectable, because of the magnitude and rapidity of this loss of collagen.

An understanding of control mechanisms of collagen breakdown would be important in the study of many biological processes including tissue growth and remodelling, embryogenesis, regeneration and involution. In the case of the uterus, the sex hormones are possible controlling agents. Rat uterine collagen undergoes significant periodic changes during the oestrous cycle (Smith & Kaltreider, 1963). Ovariectomized rats lose considerable amounts of collagen from their uteri (Harkness, Harkness & Moralee, 1956), whereas normal rats given oestrogens show an increase in the weight and collagen content of the uterus (Smith & Allison, 1966). Ovariectomy at the time of parturition leads to a great hyperinvolution and loss of uterine collagen (Morrione & Ru, 1964). In

the experiments described below, oestrogenic hormones were administered to rats just before and after parturition to see whether the rate or extent of collagen breakdown was affected. It is well known that many of the acid hydrolases in the uterus, such as β -glucuronidase (Watanabe & Fishman, 1964), acid phosphatase (Lobel & Deane, 1962), cathepsin (Rich, 1965) and β -galactosidase (Bulmer, 1964), are under oestrogenic control. Presumably these hydrolases in the uterus are contained in lysosomes. In the present studies these four hydrolases were determined quantitatively in control and oestrogen-treated involuting uteri to see whether the effects of oestrogen on collagen breakdown might be mediated through the lysosomes.

A brief description of this work was published by Woessner (1966).

METHODS

Tissue preparation. Female albino rats, about 16 days pregnant, were purchased from Sprague-Dawley Inc., Madison, Wis., U.S.A. The rats were housed in individual cages with nesting material and were maintained on Purina Chow and water *ad lib.* until used. The time of parturition was usually ascertained to within ± 3 hr. The average weight of the rats after parturition was 290 g.

The rats were anaesthetized by ether and killed by decapitation. The uteri were rapidly removed and trimmed free of mesentery, cervix and fatty nodules at the attachment sites. Uteri with less than ten foetal sites or only one pregnant horn were discarded. The uteri were slit open, rinsed in cold 0.9% NaCl, blotted, weighed on an analytical balance and then finely minced with scissors. The tissue from each uterus was transferred to a 10 mm. x 13 mm. Pyrex tube with a Teflon-lined cap, and hydrolysed at 110° for 16 hr. in 5 ml. of 6M-HCl. The hydrolysates were assayed for hydroxyproline (Woessner, 1961) to determine the collagen content, which was taken as hydroxyproline content x 7.46.

Enzyme assays. In cases where acid hydrolase activities were to be determined, the weighed minced tissue was transferred to a chilled all-glass TenBroeck homogenizer (A. H. Thomas Co., Philadelphia, Pa., U.S.A.). Then 10 ml. of cold water was added/g. of tissue, and 1 drop of Triton X-100 was included. The tissue was first ground by hand, and then the pestle was attached to a motor and five passes were made at 1500 rev./min. The resulting homogenate was centrifuged for 30 min. at 40000 g. The supernatant was used for enzyme assays, and the pellet was hydrolysed for hydroxyproline determination (Woessner, 1961). The losses of hydroxyproline in the supernatant, or of enzymes in the pellet, did not exceed 5%. Assays were performed for cathepsin D (EC 3.4.4.23), β -glucuronidase (EC 3.2.1.31), β -galactosidase (EC 3.2.1.23) and acid phosphatase (EC 3.1.3.2) by the methods of Woessner (1965). The results are expressed as μ moles of substrate hydrolysed/hr./g. wet wt. of tissue.

Determination of free hydroxyproline. Portions of the supernatant were dialysed to equilibrium against 2 vol. of

water. The diffusate was dried on the steam bath in the assay tubes. Then 2 ml. of water was added, and hydroxyproline was determined directly without hydrolysis (Woessner, 1961). Corrections for dilution and equilibrium distribution were made.

Hormone treatments. The following hormones were used: 17 β -oestradiol [oestra-1,3,5(10)-triene-3,17 β -diol] and diethylstilboestrol [3,4-bis-(*p*-hydroxyphenylethyl)hex-3-ene] supplied by Mann Research Laboratories, New York, N.Y., U.S.A., and 17 α -oestradiol [oestra-1,3,5(10)-triene-3,17 α -diol; C grade] from Calbiochem, Los Angeles, Calif., U.S.A. The hormones were finely dispersed in 0.9% NaCl by the use of a tissue homogenizer. Concentrations were such that the dose was administered in 0.1-0.2 ml. of 0.9% NaCl injected intraperitoneally.

RESULTS

Involution of control uteri. The involution of the rat uterus was followed for 8 days after parturition. The collagen hydroxyproline content and wet-weight results are presented in Table 1. These results are in close agreement with those reported by Woessner (1962, 1965). These control rats did not receive injections of any sort. However, further control experiments were performed in which rats received daily injections of the saline medium used in the oestrogen experiments. The additional treatment and handling did not affect the control values of uterine weight and collagen content significantly.

Table 1. *Effect of oestrogen treatment on the involuting rat uterus*

The results are expressed as means $\pm$ s.d., with the numbers of animals in parentheses. The day of parturition is referred to as day 0. Hormones were administered on days 0, 1, 2 and 3 only. For details see the Methods section.

Treatment	Daily dose (μ g.)	Day killed	Wet wt. of uterus (mg.)	Hydroxyproline content (μ g./uterus)
Control		0	2173 $\pm$ 429 (23)	8830 $\pm$ 741 (20)
		1	1437 $\pm$ 396 (12)	6700 $\pm$ 1440 (12)
		2	888 $\pm$ 157 (8)	4110 $\pm$ 1020 (8)
		3	668 $\pm$ 112 (21)	2410 $\pm$ 595 (16)
		4	487 $\pm$ 91 (23)	1220 $\pm$ 339 (23)
		8	315 $\pm$ 61 (15)	1080 $\pm$ 326 (15)
17 β -Oestradiol	1000	4	956 $\pm$ 170 (15)	5000 $\pm$ 889 (15)
	100	4	1040 $\pm$ 128 (21)	5750 $\pm$ 873 (21)
	100	6	842 $\pm$ 80 (9)	4710 $\pm$ 464 (9)
	100	8	927 $\pm$ 139 (10)	4700 $\pm$ 754 (11)
	100	14	401 $\pm$ 128 (7)	2280 $\pm$ 521 (7)
	10	4	825 $\pm$ 152 (14)	4350 $\pm$ 1280 (15)
	10*	8	771 $\pm$ 112 (9)	3550 $\pm$ 649 (9)
	1	4	672 $\pm$ 74 (10)	2530 $\pm$ 355 (10)
17 α -Oestradiol	100	4	769 $\pm$ 62 (8)	2930 $\pm$ 194 (8)
Diethylstilboestrol	2000	4	1075 $\pm$ 77 (8)	5340 $\pm$ 414 (8)
	100	4	846 $\pm$ 110 (6)	4250 $\pm$ 574 (6)
Not nursing		4	749 $\pm$ 161 (15)	2100 $\pm$ 535 (16)

* In this case the hormone was administered from day 0 to day 7.

Fig. 1. Effect of oestrogen treatment on collagen breakdown in the involuting rat uterus. The bars represent the amount of uterine collagen (as hydroxyproline) remaining 4 days after parturition. The indicated doses were given once a day, commencing shortly after parturition. The control-value line is extended vertically through all the bars to emphasize the excess of collagen (to the right of the line) that remained in the treated uteri. The lines at the end of the bars indicate the 95% confidence intervals. Each bar value is significantly different from all other bar values at the level $P=0.05$ or less with the exception of the pairs oestradiol (100 μ g.)-diethylstilboestrol (2000 μ g.), diethylstilboestrol (2000 μ g.)-oestradiol (1000 μ g.) and oestradiol (10 μ g.)-diethylstilboestrol (100 μ g.). (1) 17 β -Oestradiol, 100 μ g.; (2) diethylstilboestrol, 2000 μ g.; (3) 17 β -oestradiol, 1000 μ g.; (4) 17 β -oestradiol, 10 μ g.; (5) diethylstilboestrol, 100 μ g.; (6) 17 α -oestradiol, 100 μ g.; (7) 17 β -oestradiol, 1 μ g.; (8) not nursing; (9) control.

Effect of oestrogen treatment. The results of various oestrogenic-hormone treatments are summarized in Table 1. In every case, the treated uteri contained more collagen and had a higher wet weight than the controls corresponding to the same day *post partum*. The most striking effect is the inhibition of collagen breakdown produced by oestrogen treatment. This is shown most clearly in Fig. 1, which summarizes the collagen-content values at 4 days *post partum*. Four days is the time at which collagen breakdown in the control uterus is almost complete; hence this is the best time to look for inhibitory effects. A highly significant inhibition was produced by only 1 μ g. of oestradiol/day. However, the greatest inhibition of collagen breakdown was produced by 100 μ g. of oestradiol/rat/day. This must be close to the optimum dose, since treatment with lower (10 μ g.) and higher (1000 μ g.) doses gave somewhat lower results.

The finding of a lower inhibition with doses of 1000 μ g. was disconcerting. However, closer study revealed that, whereas the average weight of all rats used in these studies was 290 g., the particular rats selected for this experiment had an average weight

of only 250 g. It was then found that within both the 100 μ g.-dose group and the 1000 μ g.-dose group there was a positive correlation between uterine collagen content and rat weight. Both groups of results were fitted by the linear regression line: wt. of hydroxyproline (μ g.) = $14.8 \times$ body wt. of rat (g.) + 1310. It was then shown that there was no significant difference in the hydroxyproline content of the uterus at 4 days among the three groups given 100 μ g. of oestradiol, 1000 μ g. of oestradiol and 2000 μ g. of diethylstilboestrol (average rat weight 268 g.) when correction was made for the differences in body weight. These were the only groups of rats where deviations from the mean weight of 290 g. were sufficient to affect the interpretation of the results.

There is not an absolute requirement for 17 β -oestradiol to produce the inhibition of collagen breakdown. Diethylstilboestrol gave results almost as striking, but it required doses 10–20 times greater to achieve the same degree of inhibition as that produced by 17 β -oestradiol. 17 α -Oestradiol showed only a trace of inhibitory action; it was about 1% as effective as the 17 β form.

Removal of the pups at the time of parturition prevented lactation and slowed uterine involution. Both the collagen content and weight values for the uteri of these rats were significantly higher than those of the controls (Fig. 1 and Table 1). In view of this finding, all data from rats that failed to nurse their pups were discarded in the present experiments. In any event, a cessation of nursing would not account for any of the present results, since all treated groups showed a greater inhibition of involution than that due to removal of the pups.

Differential effects on collagen and weight loss. Fig. 2 emphasizes differences in the rates of loss of collagen and wet weight from the uterus. Initially, wet weight was lost very rapidly. Half of the weight was lost in the first 24 hr. *post partum* (bars C0 to C1). In this same period only 25% of the collagen was lost. As a result, the concentration of collagen increased in the early part of involution. This is shown by the bowing forward of the plane that represents the main sequence of involution in control rats. The maximum concentration of collagen was reached at about 36 hr. *post partum*. By 3 days the concentration decreased to a value corresponding to that at parturition, and a lower value was reached by 4 days. One consequence of the different rates of loss of weight and collagen is that any factor that inhibits involution just by slowing down all the processes uniformly will not produce an equal percentage inhibition of the two parameters. For example, if an inhibitor slowed involution such that by 4 days *post partum* the involution had progressed only to the stage equivalent to 1 day *post partum*, then collagen would be

Fig. 2. Changes in collagen concentration during uterine involution. This three-dimensional graph illustrates the changes in wet weight and collagen content of the uterus as summarized in Table 1 and further shows how the concentration of collagen is affected by different rates of loss of weight and collagen. The main sequence of uterine involution is represented by the curved planar structure. This was constructed by interpolation between the five upright bars labelled C0–C4, which correspond to the five control values at days 0, 1, 2, 3 and 4 *post partum*. The height of the bars (z axis) represents the wet weight, which starts at 2.71 g. at bar C0 and falls to 0.48 g. by bar C4. Equidistant horizontal lines along the plane continue the weight scale at 0.3 g. intervals. The sweep of the plane to the right along the x axis reflects the loss of collagen, which falls from 8.8 mg. of hydroxyproline at bar C0 to 1.2 mg. at bar C4. The y axis indicates the concentration of collagen in terms of mg. of hydroxyproline/g. wet wt. This information is not given in Table 1 but was calculated separately from values for each individual uterus. The bowing forward of the plane represents an increase in collagen concentration. The vertical bars labelled E4, E6, E8 and E14 refer to uteri of rats treated for 4 days with 100 μ g. of 17 β -oestradiol/day and then removed at days 4, 6, 8 and 14. Bar D refers to the group treated with 2000 μ g. of diethylstilboestrol/day for 4 days; bar A refers to the group treated with 100 μ g. of 17 α -oestradiol/day; bar N refers to the non-lactating group (cf. Table 1). These are discussed in the text.

lost only to the extent of 25% but wet weight would be lost to the extent of 50%.

Oestradiol treatment blocked collagen breakdown to a greater extent than it blocked other involutionary processes. It did not just retard the entire process of involution (the result of this would have been that by 4 days the uterus of the treated animal would have been like that of a control animal at 1 or 2 days). This is shown in Fig. 2 by the bars E4, E6 and E14, which all lie significantly in front of the main-sequence plane ($P=0.001$). The fact that the bars are in front of the plane means that the collagen concentration of these groups was above normal, and in fact exceeded the concentration found at any stage of normal involution. The bars are projected back on to the plane along lines representing equal collagen content. The height

of the bars represents the wet weight of the uterus. In each case (E4, E6 and E14) this weight was considerably less than the control value for the corresponding value for collagen content. One must move along the main-sequence plane for 0.5–1.0 day to the right to come to the corresponding wet-weight value. Thus it is concluded that oestradiol retards loss of collagen to a greater extent than it retards loss of weight.

The bar representing the group treated with 100 μ g. of oestradiol and killed 8 days later (bar E8) was in front of the main-sequence plane, but not significantly so ($P>0.05$). Presumably this group of uteri had a high wet weight owing to biological variability. With lower doses of oestradiol (not illustrated) the values still fell in front of the plane, but the differences are too small to be significant.

Fig. 3. Effect of 17β -oestradiol dose schedule on collagen content of involuting uterus. All rats were treated with daily doses of $100\text{ }\mu\text{g}$. of 17β -oestradiol on the days indicated *pre* and *post partum* for hydroxyproline assay. The lines at the ends of the bars indicate the 95% confidence interval. The treatment on days 0, 1, 2 and 3 gave a value for collagen content significantly higher ($P=0.05$) than did all other treatments. The untreated control gave a value significantly lower than did all other treatments. The single-dose treatments gave values significantly lower than did the optimum double-dose treatment.

The same is true for 17α -oestradiol (bar A). It is noteworthy that, whereas diethylstilboestrol ($2000\text{ }\mu\text{g}$.) blocked collagen breakdown to the same extent as did oestradiol ($100\text{ }\mu\text{g}$.), it failed to show the differential effect; instead, the bar representing this group (bar D) fell directly on the main sequence plane. This result suggests that diethylstilboestrol may block collagen breakdown by a mechanism different from that with oestradiol.

It could be argued that oestradiol might not have a differential effect on two separate involutionary processes, but might just decrease the water content of the treated uteri. This point was tested by determining the wet weight/dry weight ratios in small groups of treated and control uteri at 4 days *post partum*. No significant differences were found.

The non-lactating group (bar N) showed a selective effect of a type opposite to that of oestradiol. The bar representing this group lay a significant distance behind the plane ($P=0.05$), showing that it had a low concentration of collagen. In this case collagen breakdown was retarded to a smaller extent than was the weight loss. The resulting uterus was much larger than normal, considering that it was 4 days *post partum*, but its collagen content was lower than one would predict on the basis of its size.

Optimum time for oestrogen administration. In the first experiments, oestradiol was administered to

Fig. 4. Effect of oestradiol on free hydroxyproline in the uterus. Each point represents the average of about 12 animals. ●, Control animals; ○, animals treated with $100\text{ }\mu\text{g}$. of 17β -oestradiol/day. The vertical lines represent 95% confidence intervals. All points for oestrogen-treated are lower than the corresponding controls ($P=0.001$).

rats shortly after parturition. Additional experiments were carried out to see the effect of varying the number and time of administration of the doses. It was found (Fig. 3) that no treatment schedule was any more effective than that first tried (first bar on graph). If the dosage was decreased to two doses on consecutive days, the greatest effect was obtained by giving one dose the day before parturition and one dose shortly after parturition. None of the two-dose treatments initiated after parturition gave inhibition comparable with this effect. Addition of another dose 1 day *pre partum* to the series of four doses at 0, 1, 2 and 3 days did not further enhance the effect (not illustrated). Hence the maximum effect was obtained only when oestradiol was given from the time of parturition (four doses) or slightly before parturition (two doses). A significant retardation of collagen breakdown was found even when the last dose of oestradiol was administered as long as 3 days before parturition.

Effect of oestradiol on free hydroxyproline. In the involuting control uterus (Fig. 4) the concentration of free hydroxyproline rose to a peak at 3 days *post partum* (Woessner, 1962). It then decreased rapidly, but it was still somewhat elevated at 8 days. In the treated animals, the concentration of free hydroxyproline rose only slightly at 3 days and was lower than the control value at every time examined. The small rise is in accord with the

incomplete blocking of collagen breakdown. It is not in accord with the idea of normal breakdown offset by increased synthesis.

Further, it might be objected that Fig. 4 refers only to the concentration of free hydroxyproline. Since oestradiol also prevents the loss of wet weight, there might be a normal amount of free hydroxy-

proline diluted by more tissue. However, this is not true. On the basis of total weight of hydroxyproline/uterus there is only 33 $\mu\text{g.}$ of free hydroxyproline/uterus in the treated uterus compared with 43 $\mu\text{g.}$ /uterus in the control at 3 days *post partum*.

Effect of oestradiol on the uterine content of acid hydrolases. A study was made of changes in the amounts of enzymes presumed to be lysosomal in origin. These enzymes include acid phosphatase, β -glucuronidase, β -galactosidase and cathepsin D. A typical result is illustrated in Fig. 5. The concentration of β -galactosidase rose by about 250% during the first 4 days of involution in control uteri. It then decreased very slowly. In oestradiol-treated rats the rise was only about 100%, and the enzyme concentration was lower than that in the controls at all points examined. Essentially the same results were obtained for all four enzymes (Table 2). In almost every case there was a lower enzyme concentration in the oestradiol-treated groups than in the controls.

This agrees with the hypothesis that a retardation of involution might result from a decrease in concentration of lysosomes. However, the picture is more complex than these results suggest. If we examine β -galactosidase in terms of the total amount of enzyme in the whole uterus, a different result is obtained (Fig. 6). In the control uteri there was a continual decline in the total amount of the enzyme during involution. Thus the simplest, but probably too naive, explanation of the increase in concentration shown in Fig. 5 is that the enzyme (in the lysosomes) is conserved while other tissue elements are resorbed.

In the oestradiol-treated group (Fig. 6) during involution there is actually more β -galactosidase in the uterus of the treated animals than in that of the

Fig. 5. Effect of oestradiol on the β -galactosidase concentration in the involuting uterus. Details of the assay are given in the Methods section. ●, Controls; ○, animals given a daily dose of 100 $\mu\text{g.}$ of 17 β -oestradiol. Vertical lines indicate the 95% confidence intervals. Each point represents the mean of about 12 animals. Each oestrogen-treatment value is significantly ($P=0.001$) lower than the corresponding control value.

Table 2. *Effect of oestradiol treatment on uterine acid hydrolase concentrations*

Details of the assays are given in the Methods section. The results are expressed as means $\pm$ 95% confidence intervals with the numbers of animals in parentheses. Oestradiol (100 $\mu\text{g.}$) was administered on days 0, 1, 2 and 3 *post partum*. Enzyme units are μmoles ($\mu\text{equiv.}$) of substrate split/hr./g. wet wt. of tissue. N.S., Not significant.

Day after parturition		Enzyme concentrations		
		Cathepsin	Acid phosphatase	β -Glucuronidase
0	Control	41 $\pm$ 5 (21)	77 $\pm$ 11 (11)	17.7 $\pm$ 2.6 (21)
3	Control	134 $\pm$ 17 (25)	195 $\pm$ 44 (15)	55.4 $\pm$ 4.0 (24)
	Oestradiol	114 $\pm$ 17 (10)	127 $\pm$ 40 (10)	39.5 $\pm$ 6.0 (11)
	P	N.S.	0.05	0.001
4	Control	149 $\pm$ 13 (29)	200 $\pm$ 32 (18)	64.8 $\pm$ 7.7 (25)
	Oestradiol	99 $\pm$ 14 (7)	115 $\pm$ 21 (4)	48.6 $\pm$ 13.3 (4)
	P	0.001	0.001	N.S.
8	Control	115 $\pm$ 12 (25)	175 $\pm$ 12 (10)	53.6 $\pm$ 6.2 (23)
	Oestradiol	88 $\pm$ 7 (13)	99 $\pm$ 12 (10)	47.4 $\pm$ 11.7 (9)
	P	0.01	0.001	N.S.

Fig. 6. Effect of oestradiol on the total uterine content of β -galactosidase. Definition of symbols and other details are as given in Fig. 5. The differences are statistically significant at 3 days ($P=0.05$) and 8 days ($P=0.001$) only.

controls. The simplest explanation of the failure of the enzyme concentration to rise to the control value during involution is the failure of other tissue elements to be resorbed so that the enzyme is diluted by more tissue in the treated animals than in the controls. Again, similar results are found with the other three enzymes. An effect of oestradiol on the lysosomes would have to be more subtle than one of decreasing the concentration of lysosomal hydrolases. This is considered further below.

DISCUSSION

Effects of oestrogens on involution. The major finding of this study is that oestrogens can inhibit the breakdown of collagen in the involuting uterus. In the control rat uterus 85% of the collagen is broken down within 4 days. In the oestrogen-treated animals only 35% of uterine collagen is broken down in that period of time. Although Morrione & Ru (1964) had noted that ovariectomy accelerated collagen breakdown, the converse experiment of supplementation with oestrogen had not been tried. In fact, Grant (1965) administered oestrone to the post-partum rat and found a retardation of weight loss that agrees quite well with the present study (at 8 days); but he did not investigate the effect on collagen.

As mentioned in the introduction, various workers had shown that a depletion of oestradiol led to a loss of collagen in the non-gravid uterus, and administration of oestradiol led to a gain in collagen content. But this was generally believed to be due to effects on collagen synthesis, in keeping with the well-known anabolic effects of this hormone. However, the present study points to an inhibitory effect on degradation rather than a stimulatory effect on synthesis.

It is clearly established that free hydroxyproline in tissues arises from collagen degradation, rather than from other pathways. The evidence was summarized by Prockop & Kivirikko (1968). Of course, free hydroxyproline can also arise from the metabolic degradation of newly synthesized collagen. Hence if oestradiol retarded involution by stimulating the synthesis of new collagen to offset the rapid breakdown of pre-formed collagen, one would expect the concentration of free hydroxyproline to be even higher than the control value. Fig. 3 shows that the greatly elevated concentration of free hydroxyproline normally found in the involuting uterus is strikingly depressed by oestradiol treatment. This strongly indicates that oestradiol blocks the degradation of collagen.

Relative efficacy of oestrogens in inhibiting collagen breakdown. In the usual assays for oestrogenic potency in the rat, 17β -oestradiol is the most effective substance; oestrone and oestriol are required in 25- and 100-fold amounts respectively to give similar effects. Diethylstilboestrol is about one-tenth as effective as oestradiol (Nicol, Bilbey, Charles, Cordingley & Vernon-Roberts, 1964). In the present study, oestradiol and diethylstilboestrol display this same proportionality in their effectiveness in inhibiting collagen breakdown. This suggests that this particular effect of oestrogens is closely related to their other, more commonly known, physiological effects. On the other hand, there is a significant difference in the extent to which oestradiol and diethylstilboestrol inhibit the loss of weight during involution; the 1:10 proportionality is not maintained in this case. The inhibition of collagen breakdown by oestradiol is selectively greater than the inhibition of weight loss, whereas diethylstilboestrol inhibits the two parameters to the same extent (i.e. the concentration of collagen falls on the normal control curve; Fig. 2). Thus it is possible that the two substances might act by different mechanisms.

The dose of oestradiol needed for maximum effectiveness ($100\mu\text{g./290g. rat/day}$) seems high when one considers that oestrogenic effects have classically been demonstrated with fractions of a microgram. However, it is possible to show a highly significant effect on involution with doses of only $1\mu\text{g.}$ Moreover the classical experiments

were done with ovariectomized animals, which are depleted of oestrogen. The parturient rat has just emerged from a period when oestrogen concentrations in the body have been very high. Although the ovary has not been producing much oestrogen, the placentae and fetuses have been doing so. Hence the treatment during the post-partum period may be viewed as maintaining a high concentration of oestrogen in an organ where it would normally decrease abruptly.

The effect of nursing and non-nursing may be related to oestrogen concentration also. Nursing leads to uterine hyperinvolution and also suppresses the return of the ovary to its normal cyclic function. In non-nursing animals the oestrous cycle is rapidly restored and uterine involution stops much earlier (Dawson, 1946). Ovariectomy extends the hyperinvolution beyond that caused by nursing (Morrione & Ru, 1964). The effects of nursing on rat uterine collagen content have also been determined by Grant (1965). He found that by 5 days *post partum* the uterus of the non-nursing rat had about 80% more collagen than that of the nursing rat. This agrees closely with the present value of about 70% found at 4 days *post partum*. Grant (1965) further showed that oestrogen administration largely reversed the hyperinvolution associated with lactation, as judged by uterine weights.

Effects of oestradiol on acid hydrolases. It was shown earlier that presumed lysosomal enzymes are quite prominent in the uterus and probably play an important role in the catabolic processes of involution (Woessner, 1965; Lobel & Deane, 1962). Schwarz & Güldner (1967) demonstrated by electron microscopy that phagocytosis and intracellular digestion probably constitute a major pathway of collagen breakdown in the myometrium. This intracellular digestion presumably involves lysosomal collagenases, cathepsins, or both. In view of the well-known effects of oestradiol in controlling the concentrations of acid hydrolases in the uterus, it was hoped that an explanation of the inhibition of uterine involution and collagen breakdown could be based on changes in the concentrations of these enzymes. However, no simple explanation has emerged. There is a general decrease in the concentrations of the hydrolases after oestradiol treatment, but this seems to be a consequence of the inhibition of involution, not the cause of it. That is, the total amount of enzyme in the treated uterus is higher at any given time than that in the control uterus, but this amount of enzyme is distributed throughout a larger mass of tissue. We might expect that oestradiol would either maintain the concentrations of hydrolases (as it does in normal uteri) or cause an elevation of enzyme concentrations (as it does in castrated animals). The total hydrolase activities

in oestradiol-treated animals decrease at a lower rate than the wet weight does, but it is not clear whether this is due to a conservation of hydrolases or to an increased synthesis.

The failure of oestradiol to produce striking changes in the amounts of acid hydrolases does not eliminate the lysosomes as possible mediators of the hormonal effect. Oestradiol might modify phagocytosis, fusion of lysosomes with phagosomes, or stability of the lysosomal membranes. There is some evidence that oestradiol (Scheib, 1963) and diethylstilboestrol (Bangham, Standish & Weissmann, 1965) have a labilizing effect on lysosomes *in vitro*.

Another important possibility is that oestrogens affect the production or activity of uterine collagenase. This enzyme was described by Jeffrey & Gross (1967); it is believed to initiate the attack on the uterine collagen fibres during involution. Although an effect of oestrogens on this enzyme might account for the inhibition of collagen breakdown, it would not, of course, account for the inhibition of the other processes of involution.

General significance of inhibition by oestrogen of collagen breakdown. It might be thought that the finding of inhibition by oestrogen of collagen catabolism in the uterus is of fairly limited importance, since the uterus is a specific target tissue for oestrogen. However, the action of oestrogens on collagen is not limited to this single tissue nor to the female organism. In post-menopausal or ovariectomized women there is a high incidence of osteoporosis, which can be treated successfully by oestrogen administration (Davis, Strandjord & Lanzl, 1966). The disease is not due to inhibition of bone formation but rather to accelerated resorption; oestrogen inhibits the resorption process (Tapp, 1966). Katz & Kappas (1966) reported that oestradiol treatment in both men and women decreases the hydroxyproline content of the urine by 60% and counteracts the rise of this in the urine normally produced by parathyroid, thyroid and growth hormones. Skosey & Kappas (1967) found that oestradiol treatment inhibits the normal loss of hydroxy[¹⁴C]proline from rat skin collagen. Oestradiol also retards the loss of collagen from carrageenan granulomas in guinea pigs (Paar & Fisher, 1960). The one hypothesis that best explains these various observations is that oestrogen inhibits the metabolic breakdown of collagen.

It is possible that in some situations oestradiol may act to stimulate collagen synthesis, especially in those tissues that are usually considered targets for oestrogens. However, it seems that the increase in amount of collagen lags behind the synthesis of other proteins and the general growth of tissue (e.g. immature rat uterus; Hurley & Herrmann, 1955) and that collagen content rises in response to

the need of the enlarged tissue for greater mechanical support. It is generally impossible to distinguish between increased synthesis and decreased degradation of collagen on the sole basis of quantitative changes in total collagen content.

This investigation was supported in part by U.S. Public Health Service Research Grant AM08399 and Program Project Grant HE11035 from the National Heart Institute. The author gratefully acknowledges the capable technical assistance of Mrs Carolyn Taplin.

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