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THE *IN VITRO* EFFECT OF OESTRADIOL ON COLLAGEN METABOLISM IN METAPHYSEAL RAT BONE

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Surviving bone pieces from the femoral and tibial metaphyses of young castrated female rats have been incubated for 6 hours with concentrations of oestradiol-17-β ranging from 10^{-9} M to 3×10^{-5} M, and the *in vitro* collagen metabolism studied. The addition of oestradiol did not produce any change in the resorption patterns of bone collagen, expressed as release of hydroxyproline to the medium. Parathyroid hormone in the incubation medium increased the resorption and decreased the incorporation rate insignificantly. Addition of both parathyroid hormone and oestradiol to the incubation medium produced a significant increase in resorption and a decrease in incorporation rate as compared with the control incubations. The bone pieces incubated with oestradiol-17-β 3×10^{-5} synthesized and incorporated significantly less hydroxyproline than the control bone pieces. This is, however, a very high concentration of the hormone and the physiological significance of the observation is doubtful.

Key words: bone resorption; bone regeneration; collagen; oestradiol; parathyroid hormone

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The *in vitro* effects of oestrogens upon bone have been studied in a few works during the last decade (Stern 1969, Atkins et al. 1972, Nordin et al. 1970). All these studies have been based on some kind of tissue culture technique. They have been mostly concerned with the resorption of bone minerals, and very high concentrations of oestrogens have as a rule produced a reduced resorption rate or an inhibition of the parathyroid-induced bone resorption.

The tissue culture techniques are, however, limited to studies of foetal or neonatal bone. Great progress would therefore be made if any similar effects were demonstrable in mature bone incubated *in vitro*.

In target tissues oestrogens have been found to induce the synthesis of a specific protein *in vitro* (Katzenellenbogen & Gorski 1972, Means & O'Malley 1971, 1972). In contrast to the reduced resorption rate of the tissue culture of bone, this specific protein was synthesized at what is believed to be physiologic doses of oestrogens (Exley 1969, Brown-Grant et al. 1970). Administered *in vivo* oestradiol has been shown to be effective in reducing bone collagen resorption *in vitro*

over a wide dose range (Langeland 1975 b). The lowest doses being found effective were probably just above the physiologic. This makes it reasonable to study more thoroughly the *in vitro* effect of oestradiol in concentrations near to the physiologic.

MATERIAL AND METHODS

Young mature female rats of the Wistar/Möllegaard strain were used in experiments 1 and 3. In experiment 2, a Sprague Dawley strain of the same weight range was used. The animals were given the usual laboratory rat diet and water *ad libitum*. All rats were oophorectomized 1 week before being killed by a blow to the neck and decapitation. At oophorectomy the body weights ranged from 183 g to 210 g and at sacrifice from 206 g to 235 g. The experiments were designed for paired comparisons and accordingly all handling, treatment, incubations and analyses were run simultaneously for all samples to be compared as pairs. For one incubation series, two animals were killed. The metaphyseal bones from the upper tibiae and the lower femurs of both sides were removed and handled as described previously (Langeland 1975 a, b). All the bone pieces were pooled and thereafter divided into four portions of approximately equal weight.

Incubations and analyses were as described in detail elsewhere (Borle & Nichols 1960, Flanagan & Nichols 1969, Langeland 1975 a, b). Also the incubation medium was as described previously (Langeland 1975 b) except for the addition of different doses of oestradiol-17-β (SIGMA) solubilized in alcohol and parathyroid hormone (Para-Thor-Mone, Lilly) as follows:

Exp. 1/Medium 1 (control): 5 $^0/_{00}$ C_2H_5OH
Exp. 1/Medium 2:
oestradiol 10^{-9} M, 5 $^0/_{00}$ C_2H_5OH
Exp. 1/Medium 3:
oestradiol 10^{-8} M, 5 $^0/_{00}$ C_2H_5OH
Exp. 1/Medium 4:
oestradiol 10^{-7} M, 5 $^0/_{00}$ C_2H_5OH

Exp. 2/Medium 1 (control): 5 $^0/_{00}$ C_2H_5OH
Exp. 2/Medium 2:
oestradiol 3×10^{-5} M, 5 $^0/_{00}$ C_2H_5OH

Exp. 3/Medium 1 (control): 5 $^0/_{00}$ C_2H_5OH
Exp. 3/Medium 2:
parathyroid hormone (PTH)
(Para-Thor-Mone, Lilly)
1 USP unit per ml. + 5 $^0/_{00}$ C_2H_5OH
Exp. 3/Medium 3:
PTH 1 USP unit/ml +
oestradiol-17-β 10^{-9} M + 5 $^0/_{00}$ C_2H_5OH
Exp. 3/Medium 4:
PTH 1 USP unit/ml +
oestradiol-17-β 3×10^{-5} + 5 $^0/_{00}$ C_2H_5OH

Statistics. Wilcoxon's test for paired samples was applied to test for statistically significant differences (Wilcoxon 1945, 1947). The differences were considered significant when $2\alpha < 0.05$.

RESULTS

Most of the results from experiment 1 are summarized in Table 1 and Figure 1. The means and standard deviations are given. However, because these experiments were designed for paired comparison, the standard deviations cannot be used for estimating significant differences. Among other things, weight and age of animals differed somewhat from one paired group to another. This may influence the standard deviations, but not the evaluation of the results when studied as paired samples.

Studying Table 1, it will be observed that the incorporation rates of the bone pieces treated with oestradiol-17-β 10^{-9}, 10^{-8} or 10^{-7} M were not significantly different from controls.

In Table 2 are presented the synthesis and incorporation patterns of the bone pieces incubated with oestradiol-17-β 3×10^{-5} M. The synthesis rate was significantly reduced with this high oestradiol concentration in the medium while the percentage of synthesized collagen incorporated into the bone was unchanged, as was the percentage of total collagen in incubated bone that was passively solubilized in the incubation medium.

As can be seen from Table 1 and Figure 2 there was a greater variance in the results when studying the resorption than when looking at the incorporation. However, the differences between means are also negligible in most instances.

Likewise, it can be seen from Figure

Table 1. Collagen metabolism in rat metaphyseal bone treated in vitro *with different doses of oestradiol-17-β (experiment 1). Incorporation and resorption are given in nanomols per mg incubated collagen per hour incubation. In this table the net resorption and incorporation are given (cf. text and Figure 1). "Solubility per cent" is the percentage of the collagen in incubated bone passively solubilized in the medium. S.D. = Standard deviation.*

Treatment	*n*	Incorporation Mean ± S.D.	Resorption Mean ± S.D.	Solubility Mean ± S.D.
Control	7	0.49 ± 0.05	0.41 ± 0.17	0.152 ± 0.056
Oestradiol 10^{-9} M	7	0.57 ± 0.05	0.29 ± 0.11	0.150 ± 0.061
Oestradiol 10^{-8} M	7	0.46 ± 0.05	0.40 ± 0.19	0.152 ± 0.060
Oestradiol 10^{-7} M	7	0.54 ± 0.14	0.39 ± 0.25	0.159 ± 0.068

1 a-d, that there was no significant difference in the total release of hydroxyproline to the medium during the total incubation period, and accordingly no difference in the amount of collagen passively solubilized in the medium (Tables 1 and 2 – "Solubility"). The total hydroxyproline found in the medium derives from three different sources: 1. Active cell mediated collagen resorption. 2. Passive collagen/hydroxyproline solubilization in the medium. 3. Newly synthesized hydroxyproline not incorporated into the bone pieces (Flanagan & Nichols 1969).

The results of the present study concerning the possible "interaction" between oestradiol and PTH are summarized in Table 3 and Figure 3. As will be seen, there was an insignificant difference between control and parathyroid hormone treated bone in collagen synthesis rates. Adding oestradiol-17-β to the medium in addition to PTH reduced incorporation rates to values significantly below the results of the controls. However, none of the oestradiol PTH-treated groups present synthesis or incorporation rates significantly different from those treated with PTH alone.

There was no difference in the percentage of synthesized hydroxyproline incorporated into the bone pieces (Table 3).

The addition of PTH to the incubation medium (Figure 3) increased the resorption rate insignificantly ($0.05<2\alpha<0.10$). When oestradiol-17-β 10^{-9} M or 3×10^{-5} M was added as well, the resorption rates significantly increased compared with untreated control incubations, while there was no significant difference compared with the bone pieces treated with parathyroid hormone alone (Figure 3).

The percentage of collagen passively solubilized in the medium during the first 2–3 hours of incubation remained unaffected by the treatment given.

The different metabolic pattern of the

Table 2. Collagen metabolism in rat metaphyseal bone treated in vitro *with oestradiol-17-β 3×10^{-5} M. Values of synthesis are given in nanomol hydroxyproline per hour per mg incubated collagen.*

Treatment	*n*	Synthesis Mean ± S.D.	Per cent incorporated Mean ± S.D.	Solubility percentage Mean ± S.D.
Control	6	1.26 ± 0.10	78.4 ± 5.5	0.224 ± 0.048
Oestradiol 3×10^{-5} M	6	1.09* ± 0.16	78.5 ± 8.1	0.250 ± 0.075

* Significantly different from control ($P < 0.05$).

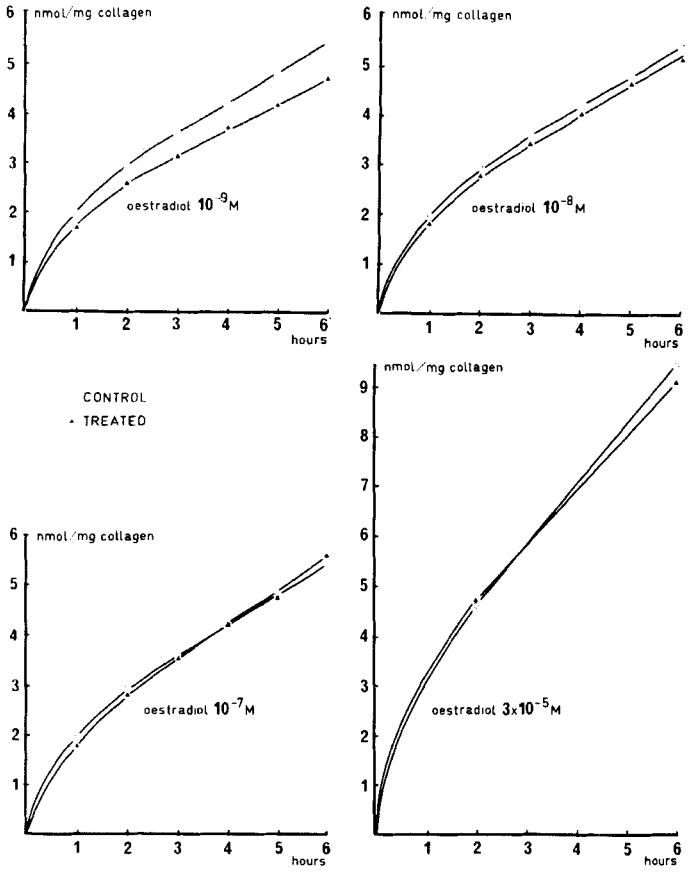

Figure 1. Total cumulative release of hydroxyproline to the medium during the 6 hours of incubation with different doses of oestradiol in the medium. Each point represents the mean of 3 to 7 samples. The standard deviations have been omitted since they overlapped each other. In each part of the figure the mean of one treated series and the corresponding untreated control series (experiments 1 and 2) are given. None of the differences between treated and corresponding control incubations were significant.

two strains of rats used in the present study should be noted.

DISCUSSION

Stimulating effects on protein synthesis of oestrogens added *in vitro* have been reported only in studies of target tissues (Katzenellenbogen & Gorski 1972, Means & O'Malley 1971, 1972). Studies on bone from mammals other than mice (Vaes & Nichols 1962, Atkins et al. 1972, Stern 1969, Nordin et al. 1970) have never revealed any stimulating effect of oestrogens on bone collagen synthesis *in vitro*. However, as pointed out in the introduction, most of these studies have been undertaken with hormone concentrations far from what is believed to be "physiologic". According to Exley (1969) the physiologic non-oestrus level of oestradiol-17-β in peripheral rat plasma is 1–2 ng per 100 ml plasma ($\approx 1–2 \times 10^{-10}$ M). It is not obvious that a similar concentration added *in vitro* is comparable. However, the doses most effective in stimulating protein synthesis in uterine tissue in the work of Katzenellenbogen & Gorski (1972) were 10^{-9} to 10^{-8} M, in other words concentrations near to the physiologic.

Based on these facts the present study was first planned with concentrations of 10^{-9} to 10^{-7} M of oestradiol-17-β. Although a slightly higher mean incorporation rate of the oestradiol 10^{-9} M group (Figure 1) can be seen, the treated bone did not behave significantly differently from control bone in any of the examined param-

Table 3. Collagen metabolism in rat metaphyseal bone treated in vitro *with parathyroid hormone and oestradiol-17-β. The total collagen synthesis, percentage of synthesized collagen incorporated into incubated bone pieces and percentage of incubated bone collagen passively solubilized in the medium are given. Values in nmol per mg collagen per hour of incubation, or in percentages.*

Treatment	*n*	Synthesis Mean ± S.D.	Per cent incorporated Mean ± S.D.	Solubility percentage Mean ± S.D.
Control	6	1.05 ± 0.17	70.4 ± 3.8	0.20 ± 0.08
Parath. hormone	7	0.99 ± 0.14	71.6 ± 4.2	0.22 ± 0.04
Parath. + oestradiol 10^{-9}	6	0.93* ± 0.13	71.1 ± 4.3	0.31 ± 0.09
Parath. + oestradiol 3×10^{-5}	7	0.89* ± 0.18	69.0 ± 2.4	0.25 ± 0.08

* Significantly different from control ($P = 0.02$).

eters. Raising the oestradiol concentration to 3×10^{-5} M reduced the collagen synthesis rate significantly, and produced an insignificant reduction in the resorption rate. This is a concentration of oestradiol-17-β comparable with the concentrations Kuchler & Grauer (1962) found to inhibit growth of L-strain fibroblasts. Atkins et al. (1972) found oestradiol to inhibit the resorptive action of parathyroid hormone at a concentration near to 10^{-4} M, and at 10^{-5} Stern (1969) found a similar effect. These experiments were performed in tissue culture.

The results obtained in the present study do not reveal any effect of oestradiol-17-β, added *in vitro*, upon bone resorption. The works of Stern (1969) and Atkins et al. (1972) revealed a clear-cut reduction in PTH-induced bone resorption at high concentrations of oestradiol.

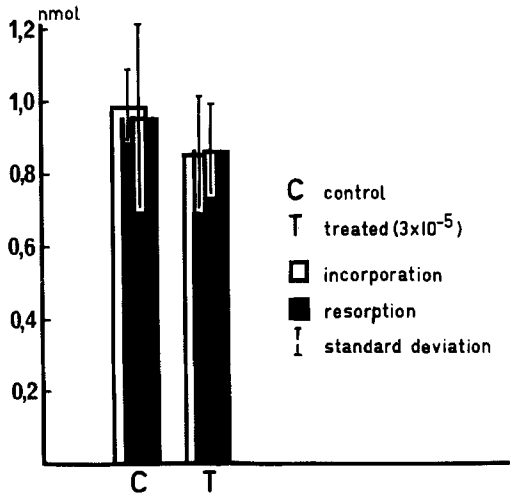

Figure 2. In vitro *treatment of metaphyseal bone from castrated female Sprague Dawley rats. The mean incorporation and resorption rates of bone pieces treated with oestradiol-17-β 3×10^{-5} M and their paired untreated controls are given. Each column represents the mean and standard deviation of 6 incubations. The treated group (T) synthesized significantly less hydroxyproline than the control incubations ($2\alpha = 0.05$).*

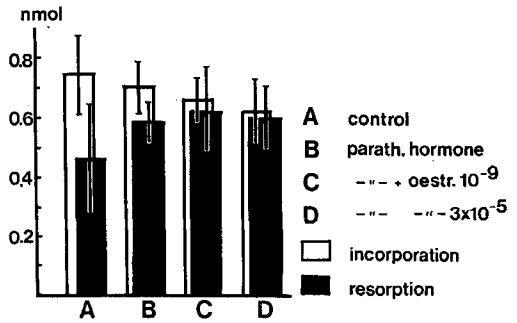

Figure 3. In vitro *metabolism of metaphyseal rat bone treated with parathyroid hormone alone and in combination with oestradiol-17-β 10^{-9} M or 3×10^{-5} M. Values are in nanomol per mg incubated bone collagen per hour of incubation. The incorporation rates of group C and D are significantly reduced compared with group A, and the resorption rates of the same groups are significantly increased as compared to group A ($2\alpha = 0.05$).*

This difference may possibly be caused by the longer incubation time possible with tissue culture techniques and by the addition of serum to the incubation medium in the cited studies (Stern & Raisz 1966, 1967). Moreover, based on the theory that oestrogens inhibit the bone resorptive activity of parathyroid hormone (Burkhardt & Jowsey 1967, Jowsey & Raisz 1968, Nordin et al. 1970), the studies of Stern (1969), Atkins et al. (1970) have been concerned with the effect of oestrogens on parathyroid-induced bone resorptions. This "oestrogen/parathyroid hormone counteraction theory" has been considered in experiment 3 of the present study. However, oestradiol-17-β in the tested concentrations had virtually no antiresorptive effect upon the bone treated with PTH. Since the techniques applied in the present study are very different from the cited tissue culture studies (Stern 1969, Atkins 1972) they are not directly comparable.

However, the concentrations found effective in reducing bone resorption *in vitro* are very high—10,000 to 100,000 times higher than what is believed to be physiologic. This does not correspond well to the results obtained with the hormone administered *in vitro* as reported previously (Langeland 1975 b), where doses, probably near to the physiologic, were found effective in inhibiting resorption of bone collagen.

As pointed out by Stern (1969) several steroids inhibit bone resorption (and bone synthesis?) when added *in vitro* at these high concentrations. She doubts that the effects of steroids at these concentrations are related to their hormonal activity. At least it seems questionable whether the effects found at these high concentrations are comparable with the effects found after *in vivo* administration of the hormone.

In conclusion, it may be pointed out that in the present study oestradiol added *in vitro* in "physiologic" amounts had no effect on bone metabolism, and it had no antiresorptive effect on PTH-treated bone. In a previous study (Langeland & Teig 1975) it was found that oestradiol administered *in vivo* had the same antiresorptive effect on bone in thyroparathyroidectomized rats as it had in animals with intact thyroparathyroid glands. All these findings suggest that the effect of oestradiol on bone is independent of PTH. However, since these effects are not demonstrable when the hormone is administered *in vitro*, the possibility that it may act indirectly, e.g., via the hypophysis or some other mediator, should be investigated further.

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