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Chemical Changes in Epiphyseal Growth Zone of Rats Induced by Excess and Lack of Estrogen.* (32367)

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There are numerous reports describing the morphologic and histochemical changes induced in the epiphyseal growth zones of young rats by administration of estrogens(1, 2). Chemical studies have usually been limited to changes in mineral content or in some single organic component of this area(1,3).

In the present study the major constituents—water, total nitrogen, collagen, mucopolysaccharides, and calcium—have been assayed in the proximal tibial growth zones of young rats and compared with similar data obtained from rats of identical age but experimentally subjected to an excess or deficit of estrogen.

Materials and methods. The experimental subjects were weanling Sprague-Dawley rats purchased from Sprague-Dawley, Inc., Madison, Wisc. For each experiment equal numbers of control and experimental rats were separately caged in clean airconditioned animal quarters. Each rat was weighed daily, and all were fed Purina rat chow and allowed water *ad lib* throughout the experiments.

In the study of the responses to excess estrogen 8 separate experiments were run using 6 controls and 6 treated rats in each. Male weanlings were used in 6 of these experiments and females in 2 of the 4 studies at the 9 dose level. Estrogen injections were started when the rats were 27-30 days old. The estrogen-treated groups received 5, 9, or 15 daily intramuscular injections of 0.1 mg estradiol benzoate in sesame oil. Both the treated rats and their controls were killed on the day following the last injection.

The effects of estrogen deficit were examined in 30 female rats ovariectomized at the age of 45-50 days and sacrificed, together with an equal number of intact female controls of the same age, at 2, 4, and 6 weeks after ovariectomy.

At termination of any given experiment each rat was exsanguinated by decapitation in a guillotine and immediately the two proximal tibial heads were taken for analysis. These samples were obtained by a uniform dissection which involved disarticulation from the femur, careful removal of adhering soft tissue, periosteum, perichondrium, and cutting of the head from the tibia at the point where the metaphysis narrows to form the shaft. Such samples, which in the present paper we have called the "epiphyseal growth zone," include the epiphysis plus the epiphyseal plate and its growing cartilage and the calcifying trabeculae of the metaphysis; *i.e.*, the epiphysis plus the components of the area which Ross and McLean have called the

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"growth apparatus"(4). Four such samples from two rats were pooled for chemical analysis.

Water content was measured by weighing each pool of fresh tissue and drying to a constant weight in an oven at 105°C.

The dry tissue was then pulverized and weighed portions were prepared for subsequent analyses. Hydrolysates obtained by heating 50 mg of the dry powder with 1 cc of 6 N HCl in a sealed glass tube for 24 hours in a boiling water bath provided, when suitably diluted, ample material for carrying out duplicate determinations of total nitrogen, hydroxyproline and calcium.

Total nitrogen was determined by micro-Kjeldahl digestion followed by nesslerization and colorimetric determination of nitrogen.

Hydroxyproline, to serve as a basis of the calculation of the amount of collagen, was determined by the method of Prockop and Udenfriend(5).

Collagen and collagen nitrogen were calculated by the factors: hydroxyproline $\times$ 7.46 = collagen, and collagen nitrogen = 18% of collagen(6,7).

Calcium was determined by EDTA titration using Cal-red as indicator(8).

Hexosamine determination appeared to furnish the most feasible index to the amount of mucopolysaccharide present. To avoid the degradation and loss of hexosamine, which was found to occur in the 24-hour hydrolysis required for complete release of hydroxyproline, it was found necessary to prepare a second hydrolysate in which 50 mg of dry powdered tissue with 1 cc of 6 N HCl was heated for only 6 hours. Hexosamine was, therefore, routinely colorimetrically determined on suitably diluted and neutralized aliquots of the 6-hour hydrolysates(9,10).

Results: Control and estrogen treated rats. During the period of rapid growth from 32-45 days normal rats increase rapidly in body weight and a rather consistent pattern of changes occurs in the chemical composition of their epiphyseal growth zones. The chemical changes which progress with increasing age are: decrease in water content, increase in the percentage of calcium, and decrease in the μg hexosamine mg collagen ratio resulting both from a gradual fall in hexosamine and a slight increase in collagen in the oldest group (Table I).

As compared to their respective controls the rats treated with estrogen showed a marked retardation in overall growth and differences in the chemical composition of the tibial heads that suggest a hastening of the normal aging process. A significant ($p=0.001$) decrease in the hexosamine/collagen ratio had already occurred in the 6 rats which had received only 5 injections of estradiol, and persisted with remarkable consistency with more prolonged treatment. This decrease in ratio was almost entirely due to a decrease in hexosamine; *i.e.,* in mucopolysaccharide, since there was no significant change in collagen content. The rats receiving 9 and 15 injections of estrogen also showed significantly ($p=0.001$) decreased water and increased calcium in their tibial-head tissue as compared to their normal controls. As a result of these various changes the 40-day-old rats which had received 9 injections of estrogen were comparable in terms of *body weight* with the 32-day-old controls, but in terms of the chemical composition of their epiphyseal growth zones they were more like the 45-day-old controls.

Result: Intact controls and ovariectomized female rats. Within 3-4 days following ovariectomy the rate of weight gain in the operated rats exceeded that of their controls, a divergence which persisted throughout the observation periods (Table II). The most consistently significant ($p=0.001$) chemical changes induced in the epiphyseal growth zone by estrogen deficit were increases in water content and decreases in the percentage of calcium. In the groups studied at 2 and 4 weeks after ovariectomy significant increases occurred in the hexosamine/collagen ratio due mainly to an increase in hexosamine with no significant change in collagen. In the few older rats in the 6-week group the significance of change in this ratio had disappeared despite a significant ($p=0.01$) decrease in collagen. Altogether the significant changes following removal of the ovaries were in the same parameters of data but consistently in the opposite direction from

TABLE I. Effects of Estrogen on Growth and Long Bone Composition in Rats.

Items	Control	Estrogen treated	P value	Control	Estrogen treated	P value	Control	Estrogen treated	P value
No. rats	6	6		24	24		18	18	
Age of rats (days)	32	32		40	40		45	45	
No. injections of 0.1 mg estradiol benzoate	0	5		0	9		0	15	
Body wt (g)	107 (±9.0)	81 (± 8.0)	.001	149 (±15.8)	109 (±15.0)	.01	162 (± 4.0)	110 (± 4.7)	.001
Water content (%)	58.6 (± .7)	57.0 (± .7)	NS	53.5 (± 2.1)	47.5 (± 3.5)	.001	51.0 (± 1.1)	41.5 (± .8)	.001
Collagen/50 mg dry tissue (mg)	6.84 (± .50)	7.13 (± .20)	NS	6.60 (± .15)	6.58 (± .23)	NS	8.40 (± .20)	8.25 (± .10)	NS
% Total N as collagen N	37.9 (±3.8)	39.0 (± .4)	NS	41.5 (± 3.7)	45.7 (± 4.5)	NS	46.3 (± 3.4)	56.3 (4.7)	.001
Hexosamine/50 mg dry tissue (μg)	500 (±5)	390 (±10)	.001	435 (±29)	339 (±37)	.001	415 (±29)	315 (±16)	.001
$\frac{\mu g \text{ hexosamine}}{\text{mg collagen}}$	73 (±5)	55 (± 2)	.005	66 (± 7)	51 (± 6)	.001	50 (± 3)	38 (± 3)	.001
% Calcium in dry tissue	16.0 (± .2)	16.4 (± .4)	NS	17.1 (± .8)	18.9 (± 1.2)	.001	18.4 (± .2)	21.8 (± .3)	.001

Figures in parentheses = standard deviation from mean.
NS = not significant.

TABLE II. Effects of Ovariectomy on Growth and Long Bone Composition in Rats.

Items	Control	Ovariectomized	P value	Control	Ovariectomized	P value	Control	Ovariectomized	P value
No. rats	12	12		12	12		6	6	
Age of rats (days)	60	60		75	75		90	90	
Body wt (g)	214 (±6)	248 (± 4)	.001	210 (±10)	256 (±17)	.001	231 (± 8)	285 (±28)	.001
Water content (%)	39.8 (±1.4)	44.8 (± 1.1)	.001	41.2 (± .1)	46.3 (± 1.0)	.001	38.4 (± 1.6)	43.1 (± 1.2)	.01
Collagen/50 mg dry tissue (mg)	7.83 (± .63)	8.19 (± .82)	NS	8.17 (± .45)	7.50 (± .61)	NS	7.37 (± .38)	6.53 (± .10)	.01
% Total N as collagen N	53.6 (±4.2)	51.7 (± 4.8)	NS	62.8 (± 5.0)	55.6 (± 1.4)	.005	66.5 (± 3.8)	57.5 (± 2.7)	.02
Hexosamine/50 mg dry tissue (μg)	253 (±5)	314 (±10)	.001	245 (± 8)	303 (± 9)	.001	253 (±15)	275 (±27)	NS
$\frac{\mu g \text{ hexosamine}}{mg \text{ collagen}}$	32 (±2.5)	38 (± 2.6)	.005	35 (± 3.0)	44 (± 2.3)	.001	36 (± 1.3)	42 (± 3.7)	NS
% Calcium in dry tissue	21.3 (± .6)	19.2 (± .9)	.001	20.7 (± .3)	18.4 (± .4)	.005	20.7 (± .1)	19.0 (± .1)	.001

Figures in parentheses = standard deviation from mean.
NS = not significant.

those induced by the administration of estrogen.

Discussion. In the present study no comparative measurements were made of the amount of food eaten by control and estrogen-treated rats. Many studies have been carried out in an attempt to explain the mechanism of the effect of estrogens both on overall growth and on individual tissue metabolism (11). Various factors, including suppression of pituitary growth hormone, decrease in food intake, metabolic alterations in the utilization of protein and other dietary constituents, or a combination of one or more of these factors have been implicated. Our consistent observations that deprivation of estrogen increased both the rate of overall growth and the mucopolysaccharide/collagen ratio in the epiphyseal growth zone, and that excess estrogen decreased both body growth and the value of this ratio, are compatible with the general concept that estrogens are antagonistic to the output or to the effects of pituitary growth hormone.

Studies in which the uptake of S^{35} has served as an index to mucopolysaccharide formation in the epiphyseal growth zone have demonstrated an acceleration of its synthesis by administered growth hormone and a retardation of S^{35} uptake in rats following hypophysectomy or following administration of estradiol or stilbestrol to intact animals(3).

In the past there has been rather general acceptance of the idea that estrogens block the output of growth hormone. With the advent of methods for the immunoassay of growth hormone in human serum it has been found(12,13) that administration of estrogens to human subjects causes a significant increase rather than a decrease of growth hormone in their sera. Such findings suggest that the antagonism between these two hormones may depend on opposing effects, as yet unrecognized, on certain metabolic processes in the target organs.

Summary. Studies have been reported on the changes induced by excess and lack of estrogen on the rate of overall growth and the chemical composition of the epiphyseal growth zone of rats as compared to control rats of the same age. The effects of estrogen administration were to retard growth and to decrease water content, increase the percentage of calcium, and to decrease the mucopolysaccharide:collagen ratio in the tissue analyzed. Deprivation of estrogen by ovariectomy had significant but opposite effects on these same parameters.

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