Nat. Cancer Inst. 31, 199 (1963).

4. Osoba, D. and Miller, J. F., J. Exptl. Med. 119, 177 (1964).

5. Law, L. W., Trainin, N., Levey, R. H., and Barth, W. F., Science 143, 1049 (1964).

6. Osoba, D., J. Exptl. Med. 122, 633 (1965).

7. Yunis, E. J., Martinez, C., and Good, R. A., Nature 204, 664 (1964).

8. Law, L. W., Nature 210, 1118 (1966).

9. Hallenbeck, G. A. and Shorter, R. G., Proc. Soc. Exptl. Biol. Med. 121, 468 (1966).

10. Kubista, T. P., Shorter, R. G., and Hallenbeck, G. A., Cancer Res. 27, 2072 (1967).

11. Sjodin, K., Dalmasso, A. P., Smith, J. M., and Martinez, C., Transplantation, 1, 521 (1963).

12. Amos, D. B., *in* "Transplantation of Tissues and Cells" (R. E. Billingham, and W. K. Silvers, eds.), p. 69. Wistar Inst. Anat. Biol. Philadelphia, Pennsylvania (1961).

13. Stutman, O., Yunis, E. J., Martinez, C., and Good, R. A., J. Immunol. 98, 79 (1967).

14. Trainin, N. and Linker-Israeli, M. Cancer Res. 27, 309 (1967).

15. Law, L. W., Goldstein, A. L., and White, A., Nature 219, 1391 (1968).

Received Nov. 18, 1968. P.S.E.B.M., 1969, Vol. 130.

Feeding Norethynodrel and Mestranol to Immature and Adult Female Rats Cross-Section and Length of Long Bones* (33739)

M. G. YANG, V. L. SANGER, AND O. MICKELSEN

Foods and Nutrition and Pathology Departments, Michigan State University, East Lansing, Michigan 48823

During the last several years, certain estrogenic compounds have been used experimentally to stop the skeletal growth rates of young girls by hastening bone maturation (1–5). The reports indicate that for girls who were treated with estradiol valerate before the onset of puberty, their bone age increased as much as 44 months after 1 year. 17-Hydroxyprogesterone caproate was used to start menstrual bleeding each month of the therapy. Progestational compounds when given to pregnant women have also been found to increase the bone age of their infants (6). Whether this was a direct action of the progestational compounds on the fetal bone is not known.

The preceding studies suggest that oral contraceptive steroid preparations containing a combination of estrogenic and progestational agents may produce comparable effects as those produced in the young girls. For this reason, norethynodrel and mestranol, the progestational and estrogenic compounds commonly used in formulating oral contraceptive pills were fed to female rats to determine whether they had any effect on the long bones. Furthermore, since prepubertal girls appeared to be more susceptible to bone growth inhibition than older girls, 5- and 12-week-old rats were used in the present experiments.

Methods. In the first of two trials, 28 virgin adult female rats, 83 days of age were paired according to body weights. Seven rats, one from each of 7 pairs were fed *ad libitum* a basal diet[1] containing 0.0225 mg of mestranol and 1.562 mg of norethynodrel/kg of diet while each pair-mate was fed a quantity of the basal diet equaling that consumed by the treated rats. The remaining 7 pairs were also pair-fed but the steroid levels were increased to twice that of the first 7 pairs. The

* Journal Article No. 4350 from the Michigan Agricultural Experiment Station, East Lansing.

[1] Percentage composition of the basal diet was: ground corn, 60.7; soybean meal (50% protein) 28.0; alfalfa meal (17% protein), 2.0; fish meal (12.5% protein), 2.5 ; dried whey (67% lactose), 2.5; limestone (38% Ca), 1.6; dicalcium phosphate (18.5% P, 22–25% Ca), 1.75; iodized salt, 0.5. Supplementary minerals and vitamins were added to provide per kg of diet: (in mg) Mn, 121; Fe, 95; Cu, 7; Zn, 4; I_2, 4; Co, 2; choline chloride, 400; Ca pantothenate, 6; riboflavin, 3; niacin, 33; menadione, 2; DL-methionine, 500; (in μg) vitamin B_{12}, 7; (in IU) vitamin A, 8010; vitamin D_2, 750; vitamin E, 5.

two levels of steroids approximated on a body weight basis the physiological doses for human adult females. In the second trial, 16 immature female rats, 35 days of age, were also paired as in the first tiral. However, only the lower levels of steroids were used since both levels in the first trial produced comparable results. The rats in both trials were Sprague-Dawley strain obtained from a commercial source when they were 3 weeks of age. They were fed the basal diet until started on the experiments.

On days 1, 14, and 28 of pair-feeding, all rats were injected subcutaneously with oxytetracycline (35 mg/kg of body wt.) to label bones according to the method described by Frost *et al.* (7). The 3 labels provided by the 3 separate injections of oxytetracycline allowed 2 measurements of bone growth of 2-week intervals. At the end of the fifth week of pair-feeding the rats of the first trial were killed whereas those in the second trial were killed at the end of 8 weeks and 3 days. The left femurs of all rats were removed and cleaned of all muscles and connective tissues. The femurs were then dried and the length measured (8). The left tibias with most of the soft tissues removed were kept in an acetate-buffer formalin solution until a cross section approximately 1.5 mm was removed from each bone 2 mm proximal to the tibio fibular junction. The cross section was ground to less than 100 μ in thickness after which the distances between the first and second oxytetracycline labels and between the second and third labels were measured. The measurements were done at $\times 200$ with an eyepiece micrometer graduated in 0.2 μ. All measurements were made at the sharp angle formed by the lateral and posterior surfaces which points directly toward the fibula. For rats in the first trial, a longitudinal section through the full width of the proximal end of the left tibia was also removed. This method is a standard bioassay for Vitamin D (9). By grinding each section to about 100-μ thick, and then staining with 5% $AgNO_3$ solution, the width of the epiphyseal line was measured at $\times 60$ with an eyepiece with lines graduated at 20 μ.

The rats were housed in individual suspended wire cages in a room with temperature maintained at 27° with lights on and off for 12 hr each. Water was given free choice. Body weights of the rats were determined at periodic intervals. All data were analyzed statistically by analysis of variance (10).

Results and Discussion. Diet and steroid consumption. In the first trial the average quantities of diet consumed during the first 5 consecutive weeks of feeding were 16.8, 15.2, 16.3, 16.4, and 17.5 g/rat/day for the rats fed the lower levels of steroids. Those fed the higher levels of steroids consumed during this period 15.4, 14.4, 16.3, 16.3, and 17.2 g/rat/day. In the second trial, the averages were 13.7, 14.7, 15.3, 15.0, and 14.8 g/rat/day. Based on concentrations of steroids in the diet, average quantities of the diet consumed and average body weights of rats during this time, the rats fed the lower levels of steroids consumed 0.0967 mg of norethynodrel and 0.0014 mg of mestranol/kg of body weight/day. Those fed the higher levels consumed 0.1911 and 0.0028 mg of norethynodrel and mestranol, respectively, /kg body weight/day. In the second trial, the steroids consumed by the rats averaged 0.1280 mg of norethynodrel and 0.0019 mg of mestranol/kg of body weight/day. These quantities of steroids are within physiological doses with the higher levels near the minimum effective level for preventing conception in rats (11).

Body weights. Since the diet containing steroids was available to the treated rats at all times, these rats presumably had certain nutritional advantages over the control rats which usually had no food during part of each day of the experiments. This was due to the control rats consuming all the allotted feed before the next feeding. Despite this and the fact that steroid-treated rats and their companion control rats consumed equal quantities of diet the control rats in both trial had greater body weights. However, the differences between control and treated rats in the two trials or between the two groups fed the two levels of steroids of the first trial were not significant, ($p > .05$). This could have

TABLE I. Average Body Weights and Some Bone Measurements of Adult Rats and Immature Rats Fed Diets with and without Contraceptive Steroids.

Measurements	First trial				Second trial	
	Level 1		Level 2		Level 1	
	Steroids[a]	Pair control	Steroids[b]	Pair control	Steroids[a]	Pair control
Body wt., initial (g)	252 ± 13	251 ± 13	251 ± 13	251 ± 13	133 ± 5	133 ± 5
Body wt., second wk (g)	262 ± 10	272 ± 14	255 ± 14	262 ± 17	167 ± 15	179 ± 13
Body wt., fifth wk (g)	271 ± 12	282 ± 15	269 ± 15	270 ± 20	193 ± 16	204 ± 11
Length, left femur (mm)[c]	34.7 ± .9	34.9 ± .7	34.4 ± .7	35.8 ± .7	31.8 ± .8	32.6 ± .4
Epiphysis, left tibia (μ)	94 ± 10	93 ± 24	91 ± 11	103 ± 27	—[d]	—[d]
Cross-section, left tibia, first 2 wk (μ)[e]	46 ± 6	61 ± 12	51 ± 10	55 ± 11	84 ± 19	91 ± 22
Cross-section, left tibia, second 2 wk (μ)[f]	32 ± 6	44 ± 3	37 ± 7	40 ± 5	67 ± 17	62 ± 14

[a] The level of steroids were 1.562 mg of norethynodrel and 0.0225 mg of mestranol/kg of diet; pair-control received equal quantities of diet without steroids; all values are averages ± SD.

[b] The level of steroids for this group of rats was 2 times that of level 1.

[c] The lengths of left femurs of rats in the second trial (immature rats) were significantly shorter for the steroid-treated rats than the companion control rats ($p < .05$). There was no significant difference in bone lengths between the steroid-treated rats and the control rats for the first trial (adult rats).

[d] No measurement taken in the second trial.

[e] The steroid-treated rats had significantly less bone growth than the pair-fed controls in the first trial ($p < .05$).

[f] The steroid-treated rats had significantly less bone growth than the pair-fed controls in the first trial ($p < .01$).

resulted from the large variations in body weights among the rats as indicated by the standard deviations (Table I).

Femur lengths and epiphysis. In the first trial wherein adult rats were used, the lengths of the femurs were not significantly reduced by feeding the steroids ($p > .05$). However, the rats in the second trial (immature rats) that received steroids had significantly shorter femurs than those of the companion control rats ($p < .05$; Table I). This was not surprising since the bones in the younger rats were still increasing in length whereas in the older rats, growth of bones was primarily limited to an increase in shaft diameter. Furthermore, a longer period of steroid feeding was used for the younger rats than the older rats. The present observations thus are in agreement with those reported for prepubertal and older girls treated with estrogenic and progestational compounds for the purpose of hastening bone maturation and preventing excessive height in these girls (1–5). The reports indicate that skeletal growth in prepubertal girls was more successfully controlled by the steroid treatment than that in postpubertal girls.

No significant difference in the widths of the epiphyseal lines was found between the steroid-treated rats and the control rats. Unfortunately, the irregularity in the outline of normal epiphyseal line introduces considerable error and there was a question whether the technique was refined enough for detecting slight changes as might be expected when physiological doses of the steroids were used. Whether epiphyseal lines from other bones might have been better sites for the determination of this is not known.

Cross sections of tibias. The cross section of the long bones is probably more important for the support of animals in an upright position than the length of these bones although obviously one is related to the other. For every change in length of the bone the effective change is decreased if it is assumed that the center of gravity for that bone is halfway between the two ends. However, for every change in the radius of the cross section the change is increased if the area of the cross section is pi times radius squared. This is a simplification of the ways stresses are endured by long bones; nevertheless, the mathematical calculations should indicate a difference not obviously recognizable when absolute changes between cross section and length of long bones are compared.

The distances between the first and second and between the second and third oxytetracycline labels represent cross-sectional growths of the tibias in 2-week intervals. In the first trial, the rats that received steroids had significantly less bone growth than that of the control rats (Table I). The growth for 4 weeks was 83 for the steroid treated rats compared to 100 μ for the control rats. The lower level of steroids used in this trial was just as effective as the higher level in reducing the cross-sectional growth of the tibia since comparable results were obtained by the two levels of steroids.

In the immature rats (second trial) the same lower level of steroids as that used in the first trial did not significantly alter the growth rate of the tibial cross section (Table I). The average total growth for 4 weeks for the immature rats was 151 μ for the steroid-treated rats compared to 150 μ for their controls. The reason for the difference in response between the immature and the adult rats to the effects of the two oral steroids is not known. Perhaps, longitudinal and cross-sectional growth of long bones proceed at different rates for the two ages and the response such as retardation of growth depends on the part of the bone that is growing most rapidly at the time of exposure to the steroids.

Summary. Norethynodrel and mestranol, a progestational and an estrogenic compound, were fed in physiological doses to half of 14 pairs of 83-day-old female rats and half of another 8 pairs that were 35 days old. The control rats were pair-fed exactly the same quantities of diet as those receiving the antiovulatory steroids. Despite the fact that the steroid-treated rats and the control rats consumed equal quantities of diet the control rats were somewhat heavier at the end of 5-weeks feeding. The 83-day-old rats treated

with steroids had significantly lesser cross-sectional growth in the tibias than their companion control rats whereas the length of the femurs and the widths of the tibial epiphyseal lines were not significantly reduced. However, in the 35-day-old rats the length of the femurs were significantly shorter in the treated rats than in the control rats.

1. Bayley, N., Gordan, G. S., Bayer, L. M., Goldberg, M. B., and Storment, A., J. Clin. Endocrinol. **22**, 1127 (1962).

2. Whitelaw, M. J. and Foster, T. N., J. Pedia. **61**, 566 (1963).

3. Whitelaw, M. J., Foster, T. N., and Graham, W. H., J. Clin. Endocrinol. Metab. **23**, 1125 (1963).

4. Greenblatt, R. B., McDonough, P. G., and Mahesh, V. B., J. Clin. Endocrinol. Metab. **26**, 1185 (1966).

5. Whitelaw, M. J., Foster, T. N., and Graham, W. H., Am. J. Obst. Gynecol. **97**, 1041 (1967).

6. Breibart, S., Bongiovanni, A. M., and Eberlein, W. R., New Engl. J. Med. **268**, 255 (1963).

7. Frost, H. M., Villanueva, A. R., Roth, H., and Stanisavljevic, S., J. New Drugs **1**, 206 (1961).

8. Sokoloff, L., Snell, K. C., and Stewart, H. L., Ann. Rheumatic Diseases **26**, 146 (1967).

9. "Official Methods of Analysis of the Association of Official Agricultural Chemists," 8th ed., p. 839. AOAC, Washington, D. C. (1955).

10. Dixon, W. J. and Massey, F. J., "Introduction to Statistical Analysis." McGraw-Hill, New York (1957).

11. Saunders, F. J., Endocrinology **63**, 561 (1958).

Received Nov. 18, 1968. P.S.E.B.M., 1969, Vol. 130.

The Partial Characterization of an Amine Oxidase in Bone Tissue* (33740)

R. B. RUCKER, J. C. ROGLER, AND H. E. PARKER
(Introduced by W. R. Featherston)

Departments of Biochemistry and Animal Science, Purdue University, Lafayette, Indiana 47907

The role of amine oxidase in the cross-linking of collagen and elastin has now been established (3, 4, 7, 12). In nutritional copper deficiency the level of this enzyme is decreased in vascular and connective tissue with a concommitant decrease in the intramolecular cross-linking of elastin (5, 7, 10) and collagen (3, 11, 15). The steps in the cross-linking mechanism are presumed to include the oxidative deamination of lysyl residues contained in collagen and elastin to residues of α-amino adipic-Δ-semialdehyde followed by aldol condensation (12). The research reported herein involves the partial characterization of an amine oxidase from bone which could catalyze the initial step of this sequence.

* Journal Paper No. 3526 (Purdue Agricultural Experiment Station, Lafayette, Indiana) supported in part by USPHS Grant No. AM-04740 from National Institute for Arthritis and Metabolic Diseases.

Materials and Methods. The femurs from 14-day-old chicks were removed, broken and immediately cleaned of adhering tissue and marrow. The bones were then placed in 0.1 *M* phosphate buffer (pH 7.4) and homogenized for 2 min at full speed in a Servall homogenizer. The homogenate (2 g of bone/10 ml buffer) was differentially centrifuged as outlined in Table I. All procedures were performed at 4°. Amine oxidase was assayed by the method of Gorkin *et al.* (4) using benzylamine as substrate, but incubated in the present experiments at 41°.

Columns of Sephadex G-100 (5× 100 cm) and Sephadex G-200 (1 × 15 cm) were calibrated for gel filtration studies as described by Andrews (1). The proteins used for calibration were purified preparations of catalase, bovine serum albumin, ovalbumin, and lysozyme (Sigma Chemicals). After calibration and equilibration of the column, 10 and 3 ml of the 110,000*g* supernatant layer