Testosterone Alters Duodenal Calcium Transport and Longitudinal Bone Growth Rate in Parallel in the Male Rat[1] (43467)

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Abstract. **Duodenal active calcium transport and longitudinal bone growth rate have been shown previously to be regulated in parallel by alteration of gonadal hormone status in sexually maturing female rats. The present study was designed to extend these observations to the sexually maturing male rat. Male rats were orchidectomized (ORX) and given Silastic implants containing either testosterone or estradiol at 6 weeks of age. At 9 weeks of age, duodenal active calcium transport was measured by the everted gut sac method and longitudinal bone growth rate was determined by tetracycline labeling. Decreases in body weight, longitudinal bone growth rate, duodenal calcium transport, and serum Ca and P were exhibited by ORX animals as compared with age-matched control animals. Testosterone administration to ORX animals resulted in an increase in body weight, longitudinal bone growth rate, duodenal calcium transport, and serum Ca and P as compared with ORX animals to a level not significantly different from that of age-matched control animals. Estradiol administration to ORX animals resulted in an additional decrease in body weight, although no significant effect on duodenal calcium transport, serum Ca, or P was noted as compared with ORX animals. There were no statistically significant alterations in the circulating levels of 1,25-dihydroxyvitamin D, parathyroid hormone, or osteocalcin in response to any of the experimental manipulations of gonadal status. These results indicate that, as in the female, gonadal hormone status affects intestinal calcium transport in sexually maturing male rats in parallel with changes in bone growth rate by mechanisms that are independent of circulating levels of 1,25-dihydroxyvitamin D.** [P.S.E.B.M. 1992, Vol 200]

Reproductive hormones have been implicated as a regulating influence on intestinal calcium transport in female rats in a number of studies involving pregnancy (1–3), lactation (1, 2, 4, 5), and aging (6, 7). Our laboratory has been interested in the alterations in calcium transport that occur during sexual maturation. In the female rat, a decline in duodenal active calcium transport occurs during sexual maturation between 6 and 12 weeks of age. This decline can be prevented by ovariectomy prior to the onset of sexual maturation (8), and the effects of ovariectomy on calcium transport can be reversed by estrogen replacement (9). It is of particular interest that the effects of ovariectomy on calcium transport occur in the absence of any change in circulating levels of parathyroid hormone (PTH) or 1,25-dihydroxyvitamin D ($1,25(OH)_2D$). However, the effects of ovariectomy on calcium transport rates are associated with increased bone growth and mineralization (9, 10).

The present experiments were designed to determine whether gonadal hormones also affect duodenal calcium transport in the male rat in a vitamin D-independent manner, and if, analogous to the situation in females, the effects of gonadal hormones on Ca transport would parallel the effects on bone growth rate. It has been documented previously in the male rat that orchidectomy results in a decreased bone growth rate which is reversible with testosterone replacement (11). We carried out preliminary experiments which

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showed that when immature, 6-week-old male rats were orchidectomized (ORX), duodenal active calcium transport remained the same as in age-matched intact animals until approximately 3 weeks after the orchidectomy. The present experiments were designed to compare the effects of orchidectomy and testosterone replacement in intestinal calcium transport, weight gain, skeletal growth rate, and $1,25(OH)_2D$ at the time when a significant change in calcium transport was first detected. In addition we wished to determine whether administration of estrogen to ORX males would decrease Ca transport as it does in ovariectomized (OVX) females, or whether the effect in females is more specifically an inhibition of ovariectomy-induced calcium transport.

In this study, in addition to measuring bone growth rate by tetracycline double labeling (see below), serum levels of osteocalcin, or bone GLA protein (BGP), were determined as an index of overall bone metabolism. Osteocalcin is a vitamin K-dependent protein synthesized by osteoblasts and found to correlate with bone turnover measured histologically (12). Osteocalcin has been shown to be elevated after ovariectomy in female rats (13). It was hypothesized that alterations in serum BGP in the male would parallel the alterations in bone growth rate.

Materials and Methods

Experimental Design and Animal Manipulation. Rats were obtained from Holtzman Co. (Madison, WI) at approximately 38–40 days of age and were acclimatized to our animal facility for a few (two to five) days before experimental manipulations were performed. At 6 weeks of age (190 g), male rats were orchidectomized under ether anesthesia. At the time of orchidectomy, Silastic capsules were placed subcutaneously in the scapular region of appropriate animals. Sham surgery was not performed because it had been determined previously that there were no significant differences between intact animals and sham-operated animals (data not shown). The rats were given free access to deionized water and a diet containing 0.5% calcium and 0.4% phosphorus (TD 83028; Teklad Test Diets, Madison, WI) for a period of 3 weeks after surgery. The animals were maintained on a 12:12-hr light:dark cycle. At 9 weeks of age, the rats were sacrificed by ether overdose, at which time blood was collected from the abdominal vena cava for serum assays and tissues were collected.

Steroid hormones were administered by way of Silastic (polydimethylsiloxane) capsules as described by Smith *et al.* (14). Silastic tubing (Dow Corning Corp., Midland, MI) was packed with crystalline hormone (i.d. 0.078 in, o.d. 0.125 in). Wooden applicator sticks were used as plugs and the capsules were sealed with silicone sealant type A (Dow Corning). The dose of the hormone delivered is determined by the surface area of the implant. The length of the implant capsule (40 mm for testosterone, 4 mm for estradiol) was designed to produce a hormone level that would approximate a normal circulating level of testosterone in males (15) and a normal circulating level of estradiol in females (16).

The results presented here reflect data collected from three separate experiments using animals that were treated by the same protocol. The data presented in Figure 1 were obtained from a single experiment using 17 animals in which longitudinal bone growth rate was measured. Separate experiments were used to evaluate bone growth and other parameters because tetracycline administration, necessary for the labeling of bone for growth measurements, may interfere with the measurement of duodenal calcium transport or serum values. The data presented in Figures 2–4 and Table I represent the pooled results obtained from two separate experiments. In one experiment containing 29 rats, intestinal calcium transport, serum Ca, P, and $1,25(OH)_2D$ were measured. In another experiment, serum Ca, P, PTH, $1,25(OH)_2D$, and osteocalcin were measured in 27 rats.

Duodenal Active Calcium Transport. Duodenal active calcium transport was measured using a modification of the everted gut sac method of Wilson and Wiseman (17), as described previously (8). The proximal duodenum was removed from each rat at the time of sacrifice. A 5-cm section of the duodenum was cleaned, everted, and ligated on one end. The sac was then filled with a transport buffer containing 0.25 m*M* Ca, ligated on the other end, and placed in a stoppered Erlenmeyer flask containing the same calcium transport buffer. The Erlenmeyer flask was incubated for 90 min in a shaking water bath, during which time the flask was gassed at 30-min intervals with 95% O_2 and 5% CO_2. After 90 min, the calcium concentration of samples taken from the flask (mucosal compartment) and from the inside of the everted gut sac (serosal compartment) was determined by atomic absorption spectrophotometry. This laboratory has shown previously that an equilibrium is established between the serosal and mucosal calcium concentrations by 60 min and that this equilibrium remains stable for an additional 60 min (8). The ratio of the calcium concentrations found in the serosal and mucosal compartments after 90 min is used as an index of active calcium transport.

Longitudinal Bone Growth Rate. Longitudinal bone growth rate was measured using the method described by Hansson (18). Oxytetracycline (Sigma Chemical Co., St. Louis, MO) was dissolved in acidified saline and administered by injection into the tail vein of ether anesthetized rats at a dose of 10 mg/kg approximately 64 hr and 16 hr before sacrifice with ether overdose. The specific time at each injection was re-

corded for each animal. The tibias from appropriately treated rats were removed and dehydrated in 70% ethanol for 1 week. The proximal end of each tibia was longitudinally sectioned using a high speed circular saw ([Dremel Tool Co., Racine, WI] with cutoff disc No. 409). The sections were soaked in acetone for 15 min to remove fat-soluble marrow elements and were then soaked in xylene for 15 min to decolorize the cartilage. The sections were dried and attached to a glass slide with rubber cement. The sections were visualized under a dissection microscope at a 40-fold magnification. The sections were illuminated by ultraviolet light and photographed through a yellow gelatin filter (Eastman Kodak, Rochester, NY). The distance between the two fluorescent bands of oxytetracycline was measured on the photograph, and longitudinal bone growth rate was calculated and expressed in micrometers per day. The data obtained agree with published data, with respect to age-matched control rats (19), gonadectomized, and gonadectomized, hormone-replaced rats (11).

Serum Assays. Serum calcium concentrations were determined by atomic absorption spectrophotometry in serum that had been diluted with a 0.5% lanthanum solution in order to eliminate interference by serum phosphates. Serum phosphorus was determined by a colorimetric assay of phosphates as described by Daly and Ertingshausen (20), using a commercially available reagent and standards (Stanbio, San Antonio, TX).

Immunoreactive parathyroid hormone was measured using a commercially available radioimmunoassay kit (Nichols Institute Diagnostics, San Juan, Capistrano, CA). This assay uses chicken antibody raised against the N-terminal(1-34) fragment of human parathyroid hormone and uses human PTH_{1-34} as standards. This assay kit has been validated for the measurement of bioactive circulating PTH in the rat (21).

Serum 1,25-dihydroxyvitamin D was measured using a commercially available radioreceptor assay kit (INCSTAR, Stillwater, MN) based on the method of Reinhardt *et al.* (22). Recovery is calculated for each sample, which is on the order of 65–75%. The coefficient of variation for this assay is 6.3% within an assay and 12.2% between assays, as reported previously (9).

Immunoreactive osteocalcin (BGP) was measured in serum using reagents available commercially (Biomedical Technologies Inc., Stoughton, MA). This assay uses goat antibody directed against purified rat osteocalcin and uses ^{125}I-rat osteocalcin as tracer.

Statistical Analyses. All data are expressed as the mean and SE for each group. Experimental data were evaluated by means of a one-way analysis of variance, using the general linear model procedure with modifications for an unbalanced design and missing values in a statistical package (SAS Institute, Cary, NC). When the F-value indicated a significant effect ($P < 0.05$), post hoc t tests were used to determine significant differences among groups.

Results

The data in Figure 1 show that at 3 weeks after orchidectomy, duodenal active transport of calcium was significantly lower than that in age-matched intact males. Estradiol treatment, at a rate which would decrease calcium transport in OVX females (9), had no effect on ORX males. However, administration of testosterone implants designed to produce a replacement concentration of testosterone returned calcium transport in ORX males to that seen in intact control animals.

The effect of sex hormone status on growth rate in male rats was evaluated by measuring both longitudinal bone growth rate at the proximal tibia and body weight. Figure 2 represents the rate of bone growth at the

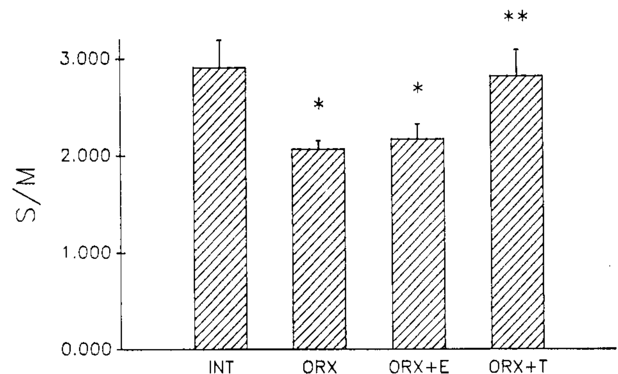

Figure 1. Duodenal calcium transport as measured by the everted gut sac method in 9-week-old male rats 3 weeks after orchidectomy and hormone implant administration. Each bar represents the mean and SE of six to eight animals. * $P < 0.05$ versus age-matched control group (INT). ** $P < 0.05$ versus ORX group. S/M, ratio of Ca concentrations found in the serosal (S) and mucosal (M) compartments; ORX + E, estradiol-treated ORX rats; ORX + T, testosterone-treated ORX rats.

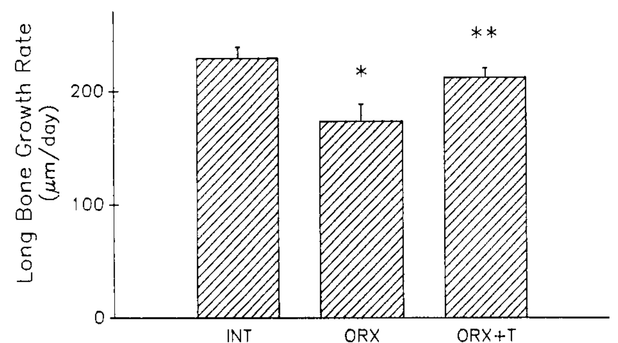

Figure 2. Longitudinal bone growth rate at the proximal tibial metaphysis using tetracycline as an intravital marker of bone in 9-week-old animals that had been orchidectomized previously and given hormone implants at 6 weeks of age, as described in Materials and Methods. Each bar represents the mean and SE of five or six animals. * $P < 0.05$ versus age-matched control group (INT). ** $P < 0.05$ versus ORX group. ORX + T, testosterone-treated ORX rats.

proximal tibial growth plate of 9-week-old rats that had been ORX or ORX and given 40-mm testosterone implants at 6 weeks of age. Orchidectomy decreased longitudinal bone growth rate compared with age-matched intact control animals, whereas testosterone replacement in ORX animals returned the growth rate to control levels. The effect of estrogen administration on bone growth rate in ORX animals was not measured.

Figure 3 represents the body weight of similarly treated 9-week-old animals with the addition of a group of rats that received 4-mm estradiol implants at the time of orchidectomy. Orchidectomized male rats weighed less 3 weeks after orchidectomy than did age-matched control animals. Testosterone-treated orchidectomized rats did not weigh more than orchidectomized rats after 3 weeks of treatment and weighed significantly less than age-matched control animals (Fig. 3). The estradiol-treated ORX group was significantly lighter than the orchidectomized rats (Fig. 3).

The wet weight of the duodenal gut sac and the wet weight to body weight ratio (GW:BW) for all groups are shown in Figure 4. Duodenal weight was obtained using the same segment of tissue used to measure calcium transport. Analysis of variance revealed a significant effect of sex hormone treatment on GW:BW ($P < 0.02$) in these animals, but not on the tissue wet weight alone. Orchidectomized rats had a higher GW:BW than age-matched control animals, as did estradiol-treated rats; these two groups were not significantly different for this parameter. There was no significant difference between testosterone-treated ORX and age-matched intact animals GW:BW.

The decrease in calcium transport resulting from ORX occurred without any statistically significant changes in circulating concentrations of $1,25(OH)_2D$ or PTH (Table I). However, the ORX and estradiol-treated ORX animals that exhibited decreased calcium transport also had decreased serum Ca and P levels relative

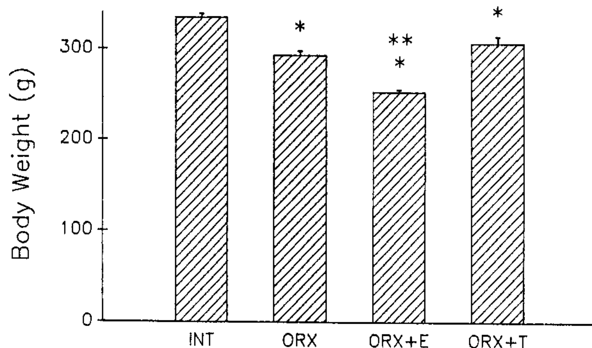

Figure 3. Body weight of male rats 3 weeks after orchidectomy and hormone implant administration. Each bar represents the mean and SE of 14 or 15 animals. * $P < 0.05$ versus age-matched control group (INT). ** $P < 0.05$ versus ORX group. ORX + E, estradiol-treated, ORX rats; ORX + T, testosterone-treated ORX rats.

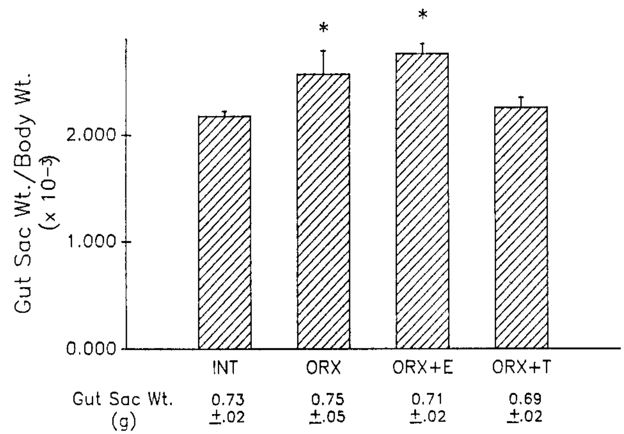

Figure 4. Gut sac weight to body weight ratio and gut sac weights of 9-week-old male rats 3 weeks after orchidectomy and hormone implant administration. Each bar represents the mean and SE of 13 to 15 animals. * $P < 0.05$ versus age-matched control group (INT). ORX + E, estradiol-treated ORX rats; ORX + T, testosterone-treated ORX rats.

to age-matched intact animals. Testosterone-treated ORX animals exhibited Ca and P levels that were not different from intact controls. There were no statistically significant differences in serum levels of osteocalcin (BGP) between any of the groups (Table I).

Discussion

These studies demonstrate that alterations in gonadal status in the sexually developing male rat alter duodenal calcium transport without any corresponding changes in circulating levels of PTH or $1,25(OH)_2D$. The present report extends to the male rat observations that had been made previously in the sexually developing female rat. Furthermore, as in the female, the effects of gonadal status on calcium transport rate in the male rat were positively correlated with the rate of longitudinal bone growth. Previous studies in this laboratory have shown that ovarian function plays an important role in the decline in intestinal calcium transport that occurs during the period corresponding to sexual development in the female rat (8). Estrogens may be responsible for the decrease in calcium transport in sexually developing female rats, because estradiol-treated ovariectomized rats exhibited decreased rates of both calcium transport and longitudinal bone growth (9). However, estradiol administration to ORX rats did not decrease duodenal active calcium transport below the level seen in ORX rats. The level seen in ORX rats may represent a baseline level of duodenal active calcium transport that cannot be further depressed. Alternatively, intestinal calcium transport in male rats may not be sensitive to the effects of estrogen.

In the present study, there were no significant alterations in serum PTH or $1,25(OH)_2D$ with alterations in testicular hormones, even though orchidectomy caused both hypocalcemia and hypophosphatemia, and

Table I. Serum Ca, P, PTH, $1,25(OH)_2D$, and BGP 3 Weeks after Orchidectomy and Hormone Treatment

	Ca (mg/dl)	P (mg/dl)	PTH (pg/ml)	$1,25(OH)_2D$ (pg/ml)	BGP (ng/ml)
Intact	11.0 ± 0.4 (n = 14)	8.9 ± 0.3 (n = 14)	31.7 ± 6.2 (n = 6)	164.3 ± 13.3 (n = 12)	85.5 ± 4.0 (n = 4)
ORX	10.1 ± 0.2[a] (n = 15)	7.9 ± 0.2[a] (n = 15)	26.8 ± 1.2 (n = 7)	145.7 ± 10.3 (n = 12)	83.3 ± 0.3 (n = 4)
ORX + E	10.0 ± 0.3 (n = 13)	7.6 ± 0.3[a] (n = 13)	33.2 ± 1.6 (n = 7)	129.9 ± 12.1 (n = 11)	95.0 ± 12.0 (n = 4)
ORX + T	10.4 ± 0.1 (n = 14)	8.8 ± 0.1[b] (n = 14)	28.9 ± 2.1 (n = 7)	170.7 ± 12.5 (n = 11)	73.9 ± 5.6 (n = 4)

[a] $P < 0.05$ versus intact group.
[b] $P < 0.05$ versus ORX group.

testosterone administration to ORX animals caused an increased in both of these parameters to a level not significantly different from that of intact control rats. Interpretation of these data must account for the finding that androgens stimulate the production of vitamin D-binding globulins (23) which alter the amount of free $1,25(OH)_2D$, which is thought to be a more relevant index of $1,25(OH)_2D$ activity (24). Data by Nyomba *et al.* (25) showed that orchidectomy, with or without testosterone administration, had no effect on the free $1,25(OH)_2D$ index, although orchidectomy decreased both vitamin D binding protein and total serum $1,25(OH)_2D$, whereas testosterone administration increased both vitamin D-binding protein and total serum $1,25(OH)_2D$. Since vitamin D-binding protein was not measured in the present experiment, it is not possible to determine whether there were alterations in the free index of $1,25(OH)_2D$ in these animals. Presumably, if orchidectomy decreased the level of vitamin D binding protein in the absence of a change in total $1,25(OH)_2D$, the circulating level of free hormone would be elevated, which would not account for the decreased calcium transport by ORX animals reported here.

In order to determine whether alterations in intestinal calcium transport reflect changes in intestinal mass in response to gonadal status, the effects of orchidectomy and testosterone on body and duodenal weights were compared. It might be expected that increases in somatic growth due to alterations in sex hormone status would be equally distributed among all parts of the body. However, sex hormone status did not alter the weight of the section of the duodenum used for calcium transport measurements. Alterations in the GW:BW seen in orchidectomized and estrogen-treated rats are due to alterations in body weight. During pregnancy and lactation in female rats, increases in calcium transport may be related, at least in part, to hypertrophic changes of the intestine, because the mucosal dry weight of both pregnant and lactating rats has been shown to be increased and the calcium transport in pregnant rats has been shown to be not different from control animals when normalized for mucosal dry weight (2). However, in the male rat, underdevelopment of the intestine, at least with respect to gross weight, did not occur with orchidectomy and testosterone treatment did not stimulate any hypertrophic increase in weight in this section of the duodenum. The alterations in intestinal calcium transport with alterations in sex hormone status in the male rat do not appear to be related to simple hypertrophic alterations in the intestinal mucosa.

Although it is known that androgens have general anabolic effects and can stimulate longitudinal bone growth (11), the effect of gonadal status on bone parameters in male rats has not been studied extensively. Wakely *et al.* (26) investigated the effect of orchidectomy and androgen administration in young, growing male rats. Tibial histomorphometry was measured 4 weeks after orchidectomy of 8-week-old rats. Orchidectomy resulted in changes at the diaphysis, including decreases in cross-sectional area, medullary area, cortical area, and cortical bone formation rate. The administration of testosterone was able to reverse the changes in cortical bone produced by orchidectomy. In spite of these skeletal responses to orchidectomy, we found no effect of gonadal status on serum levels of osteocalcin. Consistent with our findings, Turner *et al.* (27) reported no change in circulating $1,25(OH)_2D$ or $25(OH)D$ with orchidectomy.

Osteocalcin synthesis by bone cell preparations has been shown to be stimulated by $1,25(OH)_2D$ and inhibited by PTH (28, 29). Therefore, the lack of an effect of gonadal status on serum osteocalcin in the present study is consistent with the finding of no alterations in circulating levels of either PTH or $1,25(OH)_2D$. However, given the fact that longitudinal bone growth and other histomorphometric parameters are depressed in orchidectomized rats and reversed by testosterone treatment, it is surprising that there was no significant alteration in this serum marker for bone growth, especially since recently it has been shown in female rats that osteocalcin increases after ovariectomy without any alteration in serum PTH or $1,25(OH)_2D$ (13).

As in the case of the sexually developing female rat, calcium transport seems to be regulated in parallel with bone growth with both orchidectomy and testosterone administration. These alterations in calcium transport appear to be independent of circulating levels of $1,25(OH)_2D$ or PTH, as in the case of the female rat. However, it is possible that the effects of sex hormone status on calcium transport might be mediated by $1,25(OH)_2D$ if the sensitivity of the intestine to the active form of vitamin D was modulated by gonadal hormone status. The parallel association between growth and calcium transport, which has been shown previously in sexually developing female rats, and now in male rats, suggests the possibility of coordinate regulation of the intestinal acquisition of calcium and the skeletal use of calcium, either by coregulation of both processes by the same signal, or by a signal from bone to control calcium absorption.

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1. Halloran BP, DeLuca HF. Calcium transport in small intestine during pregnancy and lactation. Am J Physiol **239**:E64–69, 1980.
2. Toraason M. Calcium flux *in vivo* in the rat duodenum and ileum during pregnancy and lactation. Am J Physiol **245**:G624–G627, 1983.
3. Brommage R, Baxter DC. Elevated calcium, phosphorus, and magnesium retention in pregnant rats prior to the onset of fetal skeletal mineralization. J Bone Miner Res **3**:667–672, 1988.
4. Boass A, Toverud SU, Pike JW, Haussler MR. Calcium metabolism during lactation: Enhanced intestinal calcium absorption in vitamin D-deprived hypocalcemic rats. Endocrinology **109**: 900–907, 1981.
5. Bruns ME, Boass A, Toverud SU. Regulation by dietary calcium of vitamin D-dependent calcium binding protein and active calcium transport in the small intestine of lactating rats. Endocrinology **121**:278–283, 1987.
6. Armbrecht HJ, Zenser TV, Bruns ME. Effect of age on intestinal calcium absorption and adaptation to dietary calcium. Am J Physiol **236**:E769–E774, 1979.
7. Russell JE, Morimoto S, Birge SJ, Fausto A, Avioli LV. Effects of age and estrogen on calcium absorption in the rat. J Bone Miner Res **1**:185–189, 1986.
8. Thomas ML, Ibarra MJ. Developmental changes in duodenal calcium transport in female rats related to ovarian hormone status and growth rate. Mech Ageing Dev **37**:221–229, 1987.
9. Thomas ML, Hope WG, Ibarra MJ. The relationship between long bone growth rate and duodenal calcium transport in the female rat. J Bone Miner Res **3**:503–507, 1988.
10. Turner RT, Vandersteenhoven JJ, Bell NH. The effects of ovariectomy and 17β-estradiol on bone histomorphometry in growing rats. J Bone Miner Res **2**:115–122, 1987.
11. Jansson J-O, Eden S, Isaksson O. Sites of action of testosterone and estradiol on longitudinal bone growth. Am J Physiol **244**:E135–E140, 1983.
12. Brown JP, Malaval L, Chapuy MC, Delmas PD, Eduoard C, Meunier PJ. Serum bone gla-protein: A specific marker for bone formation in postmenopausal osteoporosis. Lancet **1**:1091–1093, 1984.
13. Ismail F, Esptein S, Fallon MD, Thomas SB, Reinhardt TA. Serum bone gla protein and the vitamin D endocrine system in the oophorectomized rat. Endocrinology **122**:624–630, 1988.
14. Smith ER, Damassa DA, Davidson JM. Hormone administration: Peripheral and intracranial implants. In: Meyers RD, Ed. Methods in Psychobiology. New York: Academic Press, Vol **3**: pp259–279, 1977.
15. Roselli CE, Resko JA. Androgens regulate brain aromatase activity in adult male rats through a receptor mechanism. Endocrinology **114**:2183–2189, 1984.
16. Bridges RS. A quantitative analysis of the roles of dosage, sequence, and duration of estradiol and progesterone exposure in the regulation of maternal behavior in the rat. Endocrinology **114**:930–940, 1984.
17. Wilson TH, Wiseman G. The use of sacs of everted small intestine for the study of transferance of substances from the mucosal to the serosal surface. J Physiol **123**:116–125, 1954.
18. Hansson LI. Daily growth in length of diaphysis measured by oxytetracycline in rabbit normally and after medullary plugging. Acta Orthop Scand [Suppl] **101**:34–107, 1967.
19. Hansson LI, Menander-Sellman K, Stenstrom A, Thorngren KG. Rate of normal longitudinal bone growth in the rat. Calcif Tissue Res **10**:238–251, 1972.
20. Daly JA, Ertingshausen G. Direct method for determining inorganic phosphate in serum with the "Centrifichem." Clin Chem **18**:263–266, 1972.
21. Toverud SU, Boass A, Garner SC, Endres DB. Circulating parathyroid hormone concentrations in normal and vitamin D-deprived rat pups determined with an N-terminal-specific radioimmunoassay. Bone Miner **1**:145–155, 1986.
22. Reinhardt TA, Horst RL, Orf JW, Hollis BW. A microassay for 1,25-dihydroxyvitamin D not requiring high performance liquid chromatography: Application to clinical studies. J Clin Endocrinol Metab **58**:91–98, 1984.
23. Bouillon R, Vandoren G, VanBaelen H, DeMoor P. Immunochemical measurement of the vitamin D-binding protein in rat serum. Endocrinology **102**:1710–1715, 1978.
24. Bikle DD, Gee F. Free and not total 1,25-dihydroxyvitamin D regulates 25-hydroxyvitamin D metabolism by keratinocytes. Endocrinology **124**:649–654, 1989.
25. Nyomba BL, Bouillon R, DeMoor P. Evidence for an interaction of insulin and sex steroids in the regulation of vitamin D metabolism in the rat. J Endocrinol **115**:295–301, 1987.
26. Wakely G, Schutte HD Jr, Hannon KS, Turner RT. Androgen treatment prevents loss of cancellous bone in the orchidectomized rat. J Bone Miner Res **6**:325–330, 1991.
27. Turner R, Wakely G, Hannon K, Bell N. Evidence that androgens are necessary for normal development and maintenance of bone [Abstract]. J Bone Miner Res **3**:S614, 1988.
28. Beresford JN, Gallagher JA, Poser JW, Russell RGG. Production of osteocalcin by human bone cells, *in vitro*: Effects of $1,25(OH)_2D_3$, $24,25(OH)_2D_3$, parathyroid hormone, and glucocorticoids. Metab Bone Dis Rel Res **5**:229–234, 1984.
29. Lian JB, Coutts M, Canalis E. Studies of hormonal regulation of osteocalcin synthesis in cultured fetal rat calvariae. J Biol Chem **260**:8706–8710, 1985.