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Control of renal vitamin D hydroxylases in birds by sex hormones

(calcium/estrogen/testosterone/bone)

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ABSTRACT **Kidney homogenates from adult male Japanese quail or chickens demonstrate hydroxylase activity predominantly for the 24 rather than the 1 position of 25-hydroxyvitamin D_3 (25-hydroxycholecalciferol). A single injection of 5 mg of estradiol-17β into a male bird completely suppresses the 24-hydroxylase and greatly increases the 1-hydroxylase activity. Immature males do not respond well to estrogen alone, but they do respond well to estradiol plus testosterone. Testosterone alone has little or no effect on the hydroxylases of either species. Castrated male chickens show an estradiol response only when testosterone is also given. Optimal 24 hr responses to 5 mg of estradiol per kg in the castrate male were obtained with about 12 mg of testosterone per kg. These optimal amounts of estradiol and testosterone increased the activity of 25-hydroxyvitamin D_3-1-hydroxylase approximately 225-fold [this enzyme is also known as 25-hydroxycholecalciferol 1-monooxygenase; 25-hydroxycholecalciferol,NADPH: oxygen oxidoreductase (hydroxylating), EC 1.14.13.13]. These results demonstrate a strong regulation by the sex hormones of the renal vitamin D hydroxylases in birds.**

There is abundant clinical evidence that sex hormones play an important role in calcium metabolism (1, 2). The widespread problem of postmenopausal osteoporosis must be related in part to an estrogen–androgen deficiency. The many attempts to define the role of estrogen in bone have indicated that it is involved in bone remodeling, but have not defined its mechanism of action (1, 2). Birds present a special case of calcium metabolism because of the necessity of shell production during the egg-laying phase of their lives. Riddle *et al.* (3) observed that serum calcium rises and calcium retention increases in female pigeons prior to the initiation of ovulation; such changes are not observed in the male. The retained calcium is deposited in medullary bone, from which it can be mobilized during egg shell formation (4). Castrated male birds given estrogen alone become hypercalcemic, but they do not retain calcium (4); whereas, when they receive both estrogen and androgen, they become hypercalcemic and retain calcium in medullary bone (4). Thus, it is clear that both estrogen and androgen are involved in calcium utilization in birds and its retention in medullary bone.

The utilization and retention of calcium and phosphorus in birds is under direct control of vitamin D (5), and vitamin D is the precursor of the hormone 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$; vitamin D_3 = cholecalciferol], which apparently directs the utilization of calcium and phosphorus (6, 7). This hormone is synthesized in the kidney from 25-hydroxyvitamin D_3 (25-OH-D_3), the major circulating metabolite of vitamin D, which is synthesized in the liver from vitamin D_3 itself. The production of 1,25-$(OH)_2D_3$ by the kidney is tightly regulated; the 25-OH-D_3-1-hydroxylase [= 25-hydroxycholecalciferol 1-monooxygenase; 25-hydroxycholecalciferol, NADPH:oxygen oxidoreductase (1-hydroxylating), EC 1.14.13.13] is stimulated by the parathyroid hormone (8, 9) and low serum phosphate (10,11) and is suppressed by phosphate and 1,25-$(OH)_2D_3$ itself (12–14). Kenny *et al.* (15) recommended the Japanese quail as an experimental animal and reported that the 25-OH-D_3-1-hydroxylase of its kidney is increased when the quail has an egg in its oviduct. Although we were unable to confirm the observation that an egg in the oviduct triggers 1,25-$(OH)_2D_3$ synthesis, we have shown that estrogen and androgen markedly stimulate the renal 25-OH-D_3-1-hydroxylase in both chicks and quail.

Abbreviations: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 25-OH-D_3, 25-hydroxyvitamin D_3; 24*R*,25-$(OH)_2D_3$, 24*R*,25-dihydroxyvitamin D_3.

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MATERIALS AND METHODS

Animals. Japanese quail (*Coturnix coturnix*) were bred and raised in the Department of Poultry Science. They were fed a corn–soy protein diet, as described by Ganther *et al.* (16). Mature quail were 4 months old, whereas immature quail were 3–4 weeks old.

One-day-old White Leghorn chicks (*Gallus domesticus*), from Northern Hatcheries, Beaver Dam, Wis., were fed an adequate chick stock diet containing the National Research Council recommended level of vitamin D_3 (17). They were used at 2 weeks as immature and 3–4 months as mature animals. Castrated chickens were purchased from Dairyland Produce, Endeavor, Wis. They were castrated at 3 weeks, and were used 4 months after the operation. They were fed the standard stock diet throughout the experiment (17).

Radioactive Materials. The 25-OH-[*26,27*-^{3}H]D_3 and 25-OH[*26,27*-^{14}C]D_3 were chemically synthesized by the method of Suda *et al.* (18). The 1,25-$(OH)_2$-[*26,27*-^{14}C]D_3 was synthesized from 25-OH-[*26,27*-^{14}C]D_3 enzymatically with chick kidney homogenate (13).

Metabolites of 25-OH-D_3. The 1,25-$(OH)_2D_3$ was chemically synthesized by the method of Semmler *et al.* (19) or was a gift from the Hoffmann-LaRoche Co., Nutley, N.J. The 24*R*,25-dihydroxyvitamin D_3 [24*R*,25-$(OH)_2D_3$] was a gift from the Hoffmann-LaRoche Co.

Hormones. The estradiol-17β valerate (Delestrogen) in castor oil and the testosterone enanthate (Delatestryl) in sesame oil were purchased from E. R. Squibb and Sons, Inc., New York, N.Y. Parathyroid extract was a gift from the Eli Lilly Co., Indianapolis, Ind. Hormones were administered subcutaneously. Sex hormones were given as a single dose 24 hr prior to sacrifice unless otherwise indicated, and parathyroid extract (50 units) was given every 8 hr for 48 hr.

***In Vitro* Incubation of Kidney Homogenate.** The measurement of the 25-OH-D_3-1-hydroxylase and 25-OH-D_3-24-hydroxylase were performed as described by Gray *et al.* (20) and Knutson and DeLuca (21) as modified by Tanaka *et al.* (13). A 20% (wt/vol) homogenate of chick kidney was prepared in ice-cold buffer (pH 7.4) containing 0.19 M sucrose, 15 mM Tris acetate, and 1.9 mM magnesium acetate. The incubations were carried out in duplicate in 25 ml Erlenmeyer flasks con-

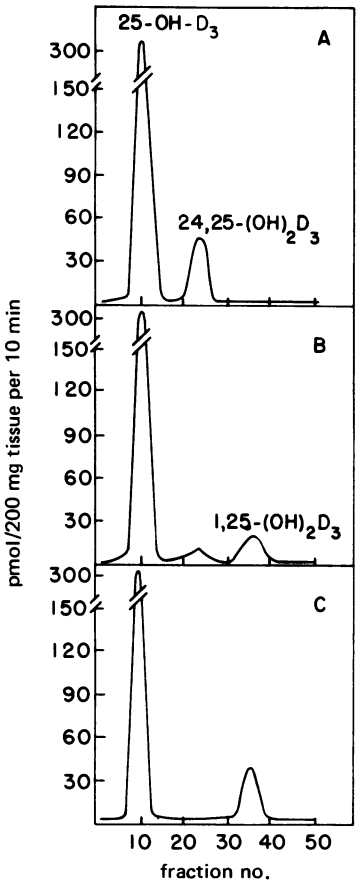

FIG. 1. Stimulation of 25-OH-D_3-1-hydroxylase by estrogen. Homogenates were prepared from pooled kidneys from three quail in each group. The homogenates were incubated with 25-OH-[*26,27*-^{3}H]D_3 as described in the *text*, in duplicate, and the lipid extracts of the incubation mixtures were chromatographed on an 8 g Sephadex LH-20 column prepared and eluted with 65% chloroform–35% Skellysolve B. In each case, 2.8 ml fractions were collected. The ^{3}H in each fraction was determined by liquid scintillation spectrometry. The identity of each metabolite was determined by high-pressure liquid cochromatography as described in the *text*. This experiment has been repeated four times with identical results. (A) Homogenate from mature male Japanese quail, (B) homogenate from laying quail, and (C) homogenate from mature male quail given estradiol (5 mg per quail) 24 hr prior to sacrifice.

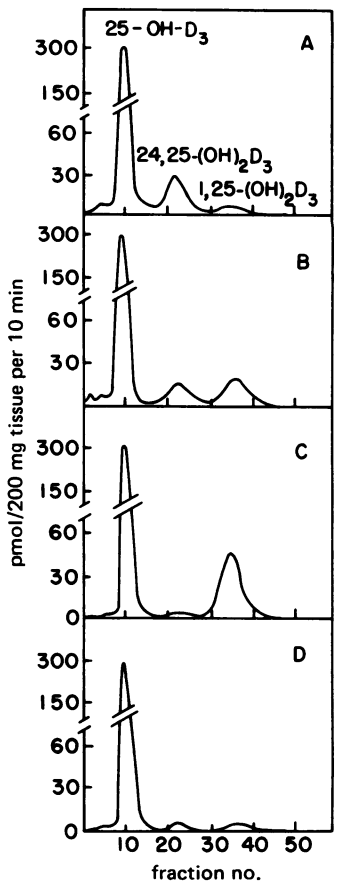

FIG. 2. Stimulation of renal 25-OH-D_3-1-hydroxylase and suppression of 25-OH-D_3-24-hydroxylase by estrogen and testosterone in immature male chickens. Experimental details were as described in Fig. 1 and in the *text*. Typical Sephadex LH-20 column profiles are plotted for (A) homogenates from immature male (2-week-old) chickens, (B) homogenate from immature male chickens given 5 mg of estradiol 24 hr before, (C) homogenate from immature male chickens given estradiol (5 mg) and testosterone (10 mg) 24 hr before, and (D) homogenate from immature male chickens given 10 mg of testosterone 24 hr before. This experiment has been repeated five times.

taining 1 ml of kidney homogenate (200 mg of kidney tissue per ml) and 0.5 ml of 75 mM succinate. The flasks were flushed for 30 sec with 100% oxygen, and the substrate 25-OH-[*26,27*-^{3}H]D_3 (1.3 nmol) dissolved in 10 μl of ethanol was added to each flask. The flasks were incubated at 37° for 10 min. The reaction was stopped by addition of 2:1 methanol–chloroform mixture and extracted as described by Lund and DeLuca (22).

Chromatography. The extracts were chromatographed on an 8 g Sephadex LH-20 column (1 × 21 cm), packed and eluted with 65% chloroform–35% Skellysolve B. An aliquot of each fraction in a toluene fluor solution (23) was counted for ^{3}H in a Packard scintillation counter (model 3375).

Identification of Metabolites. Material believed to be 1,25-$(OH)_2D_3$ was obtained in each experiment from Sephadex LH-20 chromatography and then was cochromatographed with chemically synthesized 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2$-[*26,27*-^{14}C]D_3 by high-pressure liquid chromatography (DuPont 830 apparatus) on a Zorbax SIL (0.79 cm cm × 25 cm) column eluted with 15% isopropanol in Skellysolve B at a pressure of 1000 pounds/inch^2 (6.9 MPa); this gave a flow rate of 3.2 ml/min (24). The 254 nm ultraviolet monitor was used to detect chemically synthesized 1,25-$(OH)_2D_3$. The radioactivity in each fraction was determined by liquid scintillation spectrometry. Fractions believed to contain 24,25-$(OH)_2D_3$ were cochromatographed with chemically synthesized 24*R*,25-$(OH)_2D_3$ as above. Another portion of these 24,25-$(OH)_2D_3$ fractions was treated with periodate solution as previously described (25); 90% of the tritium label at C-26 and C-27 was lost following periodate oxidation (25). The suspected 1,25-$(OH)_2D_3$ was not sensitive to periodate oxidation.

RESULTS

Fig. 1 demonstrates that mature male Japanese quail on an adequate stock diet have predominantly 25-OH-D_3-24-hydroxylase in their renal tissue, whereas the mature female quail in the egg-laying stage have mostly 25-OH-D_3-1-hydroxylase activity. Similar data were obtained with mature chickens. Although it has been suggested that the 25-OH-D_3-1-hydroxylase is stimulated by the appearance of the egg in the oviduct (15), we have been unable to confirm this point. Regardless of the position of the egg is the oviduct, the mature laying quail

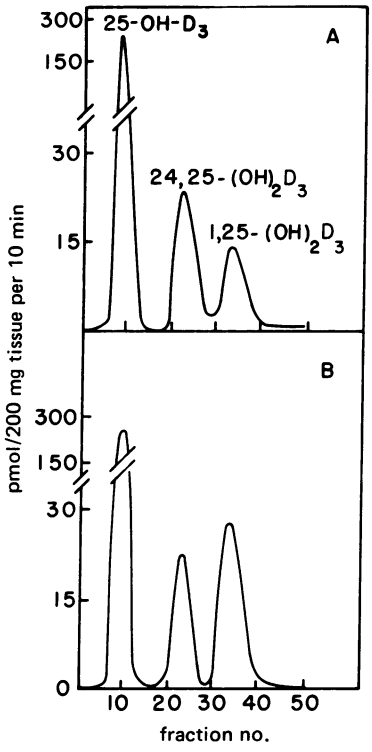

FIG. 3. Effect of estradiol and testosterone on the renal vitamin D hydroxylases of immature female chickens. The extraction and chromatography were carried out as described in Fig. 1. (A) Immature (2-week-old) female chickens and (B) immature females given estradiol (5 mg) and testosterone (10 mg) 24 hr before.

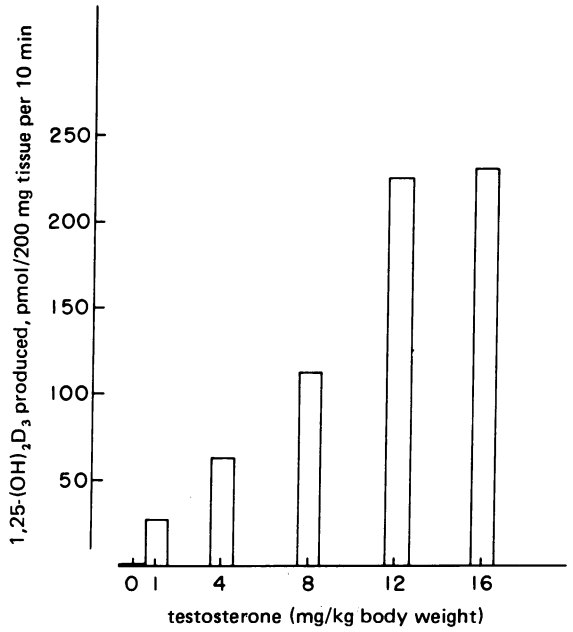

FIG. 4. Testosterone dependence of estradiol stimulation of renal 25-OH-D_3-1-hydroxylase of castrate male chickens. Incubations, extractions, and chromatography were carried out as described in the *text* and in Fig. 1. Castrate males (4-month-old) were all given 4 mg/kg of estradiol. The animals received the indicated testosterone dose, and 24 hr later their kidneys were homogenized to determine the renal 25-OH-D_3-1-hydroxylase activity. The renal 1-hydroxylase activity is plotted versus testosterone dose.

always showed enhanced 25-OH-D_3-1-hydroxylase as compared to mature male quail. Inasmuch as an important hormonal difference between these animals on identical diets is the estrogen level, mature male quail were given estrogen 24 hr prior to measurement of the hydroxylases. Fig. 1C demonstrates that estrogen administration markedly stimulates the 25-OH-D_3-1-hydroxylase and suppresses the 24-hydroxylase in the mature male quail. A similar picture was observed for chickens given estradiol.

When the same estradiol treatment was given to immature male chickens, an enhancement of the 25-OH-D_3-1-hydroxylase was observed (Fig. 2B), but the degree of enhancement was substantially below that found in the mature male (Fig. 1C). Since the diet and circumstances of both the mature and immature male chickens were identical, it appeared that the male sex hormones might also be involved. The results of administration of both estradiol and testosterone to immature male chickens (Fig. 2C) illustrate that the presence of an androgen (testosterone) provides a marked increase in the stimulation of 25-OH-D_3-1-hydroxylase by estradiol. Fig. 2D illustrates that testosterone alone had little effect on the renal hydroxylases of the immature chicken, although some suppression of the 25-OH-D_3-24-hydroxylase was noted. In immature female chickens (Fig. 3), the 24-hydroxylase was not suppressed to the degree found in immature male chickens (Fig. 2C) by the treatment with both testosterone and estradiol, although there was a clear stimulation of the renal 25-OH-D_3-1-hydroxylase.

Because of the difference in sensitivity of the mature versus immature as well as of the male versus female birds to these sex hormones, an experiment was performed with castrate male chickens to eliminate the effect of endogenous testosterone (Fig. 4). All of these animals were given 4 mg of estradiol per kg. Increasing doses of testosterone from 1 to 16 mg/kg were also given. Estradiol at 4 mg/kg without testosterone did not stimulate the 25-OH-D_3-1-hydroxylase in the castrate male, at least at this dosage level. However, the estrogen produced a clear response when testosterone was given even at 1 mg/kg. This response was increased dramatically by greater doses of testosterone up to 12 mg/kg; this level increased the 25-OH-D_3-1-hydroxylase 225-fold. Fig. 5 demonstrates that the immature male quail responds to parathyroid hormone by increasing its renal 25-OH-D_3-1-hydroxylase. Testosterone–estrogen also increased the 1-hydroxylase, and when parathyroid extract and the estrogen–testosterone were given together, the enhancement of the 1-hydroxylase was additive.

DISCUSSION

It is of interest that estrogen can markedly increase the biosynthesis of the active form of vitamin D, inasmuch as an estrogen deficiency has been linked to the metabolic bone disease, osteoporosis. There is no doubt that 1,25-$(OH)_2D_3$ enhances bone formation, bone turnover, and bone resorption, although the bone resorption response also requires the presence of parathyroid hormone *in vivo* (26). The relationship between the sex hormones and 1,25-$(OH)_2D_3$ synthesis may give us insight into the etiology of osteoporosis and its relationship to estrogen deficiency. However, these studies were carried out in birds, which have unusual calcium metabolism related to egg shell formation. Thus, it is possible that the stimulation by estrogen of 1-hydroxylase may apply only to shell-forming animals. At the very least, the present study may indicate a metabolic basis for the formation of medullary bone and for other changes in calcium metabolism in birds in preparation for egg production. It will be of interest to study mammalian species to determine whether or not the sex hormones play a controlling

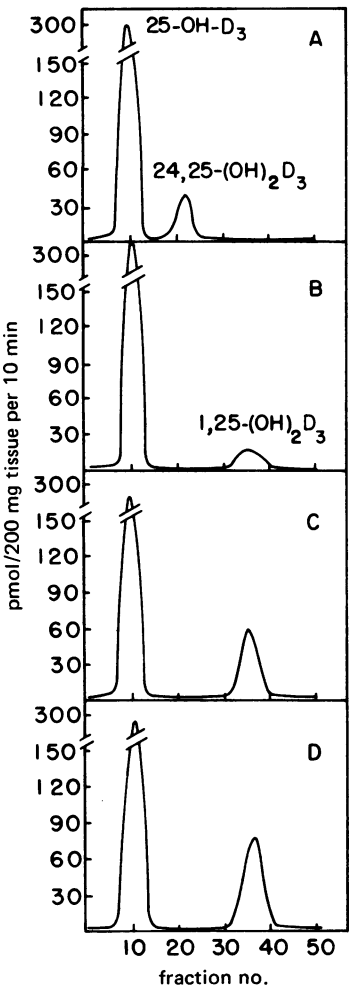

FIG. 5. Sephadex LH-20 chromatographic profiles of chloroform extracts from incubations of Japanese quail kidney homogenates. (A) Kidney homogenate of mature male quail, (B) kidney homogenate of mature male quail given 50 units parathyroid extract each 8 hr beginning 48 hr prior to sacrifice, (C) kidney homogenate of mature male quail given 5 mg of estradiol and 10 mg of testosterone 48 hr prior to sacrifice, and (D) kidney homogenate of mature male quail given 5 mg of estradiol and 10 mg of testosterone 48 hr prior to sacrifice and given 50 units parathyroid extract each 8 hr starting 48 hr prior to sacrifice. Kidneys from two to three quail in each group were combined and incubations were carried out in duplicate.

role in the vitamin D hydroxylases in species that do not form egg shells.

Our results demonstrate that an androgen (testosterone) must be added for estrogen to stimulate the 25-OH-D_3-1-hydroxylase in immature or castrate birds, whereas mature males respond well to estrogen injections alone. Thus, it appears that androgens act as a permissive agent for estrogen stimulation of 1,25-$(OH)_2D_3$ synthesis. This may help to explain why estrogen alone will not stimulate medullary bone formation, whereas estrogen plus androgen will. It is of interest that both estrogen and androgen levels are low in postmenopausal women (27).

As suggested by Kenny *et al.* (15), the Japanese quail is an excellent experimental model for studying the vitamin D hydroxylases. They are less expensive to maintain than chicks and are highly responsive to hormonal stimulators of the vitamin D hydroxylases, including parathyroid hormone.

The mechanism whereby the sex hormones stimulate the 25-OH-D_3-1-hydroxylase is unknown, as is its exact physiologic significance. However, this newly discovered relationship between sex hormones and calcium metabolism may furnish insight into how changes in sex hormone levels may influence calcium metabolism and metabolic bone diseases.

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