Chemical Synthesis, Biological Activity, and Metabolism of 25-Hydroxy-24-oxovitamin D₃*

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25-Hydroxy-24-oxovitamin D₃ (25(OH)24-oxo-D₃), a metabolite of 25-hydroxyvitamin D₃, has been chemically synthesized. The ultraviolet, mass, infrared, and proton nuclear magnetic resonance spectra of the 25(OH)24-oxo-D₃ were identical with those of the natural product isolated from chick kidney incubates. The oxo compound showed biological activity similar to 24,25-dihydroxyvitamin D₃ (24,25(OH)₂D₃) in vitamin D-deficient chicks in enhancing intestinal calcium transport and bone calcium mobilization activities. Although 25(OH)24-oxo-D₃ partially restored the impaired eggshell weights of Japanese quails fed a vitamin D-deficient diet, it was much less potent than 25-hydroxyvitamin D₃, or 1α,25-dihydroxyvitamin D₃. In addition, there was no effect on the calcification of modulary bone. When 25(OH)24-oxo[3H]D₃ was incubated with kidney homogenates from vitamin D-deficient chicks, it was metabolized to [3H]-1α,24,25-trihydroxyvitamin D₃ and a metabolite which was eluted in a region between authentic 2α,25(OH)₂D₃ and 1α,25-dihydroxyvitamin D₃ on high pressure liquid chromatography. In the incubates of kidney homogenates from vitamin D-supplemented chicks, those metabolites were not detected. In vitamin D-supplemented chicks, the recovery of radioactivity in the chloroform phase produced in the kidney (1) and intestine (2) and possibly in the cartilage (3) by a mixed-function oxygenase (4). Its production is controlled by the hormonal form of vitamin D₃.

Recently much attention has been focused on the biological significance of 24,25-dihydroxyvitamin D₃. This metabolite is produced in the kidney (1) and intestine (2) and possibly in the cartilage (3) by a mixed-function oxygenase (4). Its production is controlled by the hormonal form of vitamin D₃.

1α,25-Dihydroxyvitamin D₃ (5, 6). 24,25(OH)₂D₃ has significant biological activity in rats (7) and chicks (8), but it is less active in chicks than in rats, probably due to more rapid metabolism in birds (9). 1α-Hydroxylation appears to be required for its activity in stimulating intestinal calcium absorption and bone mineral mobilization in rats (7). In addition, 24,25(OH)₂D₃ has been reported to have specific action in the mineralization of bone (10), proteoglycan synthesis of cartilage (11), suppression of parathyroid hormone secretion (12, 13), and in chicken egg hatchability (14).

DeLuca and Schnoes, on the other hand, demonstrated that a substantial amount of 24,25(OH)₂[3H]D₃ was metabolized very rapidly to water-soluble compounds when 24,25(OH)₂D₃ was administered to normal rats (15). They isolated a new metabolite from the aqueous phase of kidney incubates with 24,25(OH)₂D₃ from normal rats given vitamin D and identified it as 2α,24,25-trihydroxyvitamin D₃-24-carboxylic acid (15).

We have also found 3 new metabolites which are synthesized in vitro from 25-hydroxyvitamin D₃ via 24,25(OH)₂D₃ in the kidney of chicks supplemented with vitamin D (16). One of those metabolites was isolated by chick kidney incubates with 25(OH)D₃ and identified as 25-hydroxy-24-oxovitamin D₃ (17). However, we could not establish the biological significance of the oxo compound since most of the isolated metabolite was used for identification.

25(OH)24-oxo-D₃ has now been synthesized chemically in our laboratory (18). This paper describes the synthesis, biological activity, and metabolism of the metabolite in birds.

MATERIALS AND METHODS

General Procedures—The ultraviolet spectra were measured with a Union Giken Model SM-401 spectrophotometer. Mass spectra were obtained by using a JEOL JMS-D300 mass spectrometer. All spectra were run at 70 eV with a source temperature programmed in the range of 50-300 °C/min. The infrared spectra were recorded on a JASCO A-302 infrared spectrometer. The proton nuclear magnetic resonance spectra were run on a Varian XL-100 spectrometer, and the chemical shift values were reported in parts per million downfield from internal Me₄Si.

Synthesis—Synthesis of 25(OH)24-oxo-D₃ was performed starting...
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from 22-phenylsulfonyl-23,24-bisanor-5,7-choladien-3β-ol, 3-tetrahydroxypropanyl ether (I in Fig. 1) and 2-(1-hydroxy-1-methylethyl)oxirane (II in Fig. 1) in 6 steps in 15% overall yield as outlined in Fig. 1. The sulfone (I) was synthesized from ergosterol by the established method (19, 20) in 24% yield and the epoxide (II) was readily obtained from commercially available 3-methyl-1-buten-3-ol by epoxidation (21). The sulfone (I) was reacted with the epoxide (II) in the presence of lithium disopropylamide at -20 °C to give the triol derivative (III) in 85% yield. Reductive removal of the phenylsulfonyl group (Na-Hg, MeOH, Na₂HPO₄) afforded the 24,25-dihydroprovitamin D derivative (IV) in 93% yield. Oxidation of the 24-hydroxyl group was achieved in good yield (80%) by using the modified Moffat method (22) dimethyl sulfoxide, pyridine-SO₃, Et₃N) to afford the 24-oxo derivative (V) which upon treatment with acidic alcohol gave the desired provitamin D (VI). The provitamin D (VI) was transformed into 25(OH)24-oxo-D₃ by the usual method of UV irradiation followed by thermal isomerization. The spectral properties, UV (95% ethanol)
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**III** (100 mg, 85% yield); IR (HCl) 1300, 1155 cm⁻¹; mass spectrum m/e 640 (M⁺), 622.

5,7-Cholestadiene-3,24,25-tri-ol 3-Tetrahydropranyl Ether (IV)—To a solution of III (160 mg, 0.25 mmol) in 2 ml of methanol were added anhydrous Na₂HPO₄ (1 g) and 5% sodium amalgam (3.2 g) at room temperature under argon. The mixture was stirred at room temperature for 30 min and then diluted with methanol (20 ml). The solid was filtered and rinsed with methanol. The filtrate was evaporated and the residue was dissolved in ether. The residue was evaporated under argon for 4 min. The residue was chromatographed on a silica gel column with 1:1 hexane/ethyl acetate (200 ml) through a Vycor filter for 3 min under argon. The residue was dissolved in 200 ml of 20% ethyl acetate in hexane, chromatographed on a silica gel column with 5:2 hexane/aqueous NaHC₀₃, and brine, dried over Na₂SO₄, and evaporated. The residue was chromatographed on a silica gel column with 1:1 hexane/ethyl acetate (200 ml) through a Vycor filter for 4 min under argon. The residue was chromatographed on a silica gel column with 1:1 hexane/ethyl acetate to give VI (36 mg, 96% yield): mp 161-163 °C (from methanol); IR (CHCl₃), 1705 cm⁻¹; NMR (CDCl₃), δ 6.02 (3H, s), 0.94 (3H, s), 1.38 (6H, s); mass spectrum, m/e 414.3112 (Found 414.3112) for 25(OH)₂4-oxo-D₃ (VIII)

**RESULTS**

**Biological Activity of 25(OH)24-oxo-D₃**—The time course of change in intestinal calcium transport response in vitamin D-deficient chicks to 6.5 nmol of 25(OH)24-oxo-D₃ or 24R,25(OH)₂D₃ is shown in Fig. 5A. Both compounds exhibited a significant response at 6 h and attained a maximum at 12 h. The maximal stimulation by 25(OH)24-oxo-D₃ was maintained up to 48 h. At 72 h the response to both compounds began to decline. Fig. 5B illustrates the time course of change in bone calcium mobilization response to 25(OH)24-oxo-D₃ or 24R,25(OH)₂D₃ in vitamin D-deficient chicks. Both compounds showed a marked rise in serum calcium levels as early as 3 h, and attained near maximum at 6 h. The maximal response to both compounds was maintained up to 48 h. No significant differences appeared in this study between 25(OH)24-oxo-D₃ and the 24R,25(OH)₂D₃ responses.

The dose responses of intestinal calcium transport and bone
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Fig. 5. Time course of response of intestinal calcium transport (A) and bone calcium mobilization (B) to 6.5 nmol of 25(OH)₂4-oxo-D₃ (■) and 24R,25(OH)₂D₃ (○). The metabolites were given intravenously. Values are the means ± S.E. of 6 to 10 chicks.

Calcium mobilization activities to 25(OH)₂4-oxo-D₃ are shown in Fig. 6. Responses were measured 24 h after intravenous administration of the metabolite. Intestinal calcium transport began to increase with doses of 0.65 nmol and attained a maximum at 19.5 nmol. Although at 1.95 nmol, 24R,25(OH)₂D₃ induced a slightly higher response than 25(OH)₂4-oxo-D₃, the difference was not statistically significant. The same dose of 25(OH)D₃ caused a higher response (Fig. 6A). Much larger amounts (6.5 nmol) of the oxo compound were required to induce a significant response of bone calcium mobilization (Fig. 6B). The bone mobilization response to 1.95 nmol of 24R,25(OH)₂D₃ and 25(OH)D₃ was almost identical with that to 6.5 and 65 nmol of 25(OH)₂4-oxo-D₃, respectively. These dose-response studies may suggest that 25(OH)₂4-oxo-D₃ is slightly less active than 24R,25(OH)₂D₃.

Fig. 7 shows the effect of various metabolites of vitamin D₃ on the eggshell weights of Japanese quails fed a vitamin D-deficient diet. After a 3-week vitamin D-depletion period, the rate of egg production decreased from 63.2 to 23.9%. In addition, the eggshell weight decreased from 0.53 ± 0.03 to 0.25 ± 0.02 g. Then the birds were separated into 5 groups and each group was given daily doses of 1.95 nmol of one of the vitamin D₃ metabolites for 4 days. The impaired eggshell weights recovered to normal level as early as 2 days after 1α,25(OH)₂D₃ administration. 25(OH)D₃ also restored the impaired weights to normal levels 4 days after administration. 24R,25(OH)₂D₃ and 25(OH)₂4-oxo-D₃ restored the weights only partially. The effect of 25(OH)₂4-oxo-D₃ on the recovery of the impaired eggshell weights was almost equivalent to that of 24R,25(OH)₂D₃.

The effects of various metabolites of vitamin D₃ on the plasma calcium levels and the calcium and phosphorus content...

FIG. 6. Dose response of intestinal calcium transport (A) and bone calcium mobilization (B) to 25(OH)₂4-oxo-D₃ (■) 24 h after intravenous administration. Response to 1.95 nmol of 24R,25(OH)₂D₃ (○) and 25(OH)D₃ (×) 24 h after intravenous administration is also shown. Values are the means ± S.E. of 6 to 10 chicks.

FIG. 7. Influence of metabolites of vitamin D₃ on the eggshell weight. Forty quails were maintained on a vitamin D-deficient diet from day 0 to day 25. The quails were separated into 5 groups on day 21 and every day for 4 days each group was given 1.95 nmol of 25(OH)₂4-oxo-D₃ (■), 24R,25(OH)₂D₃ (○), 25(OH)D₃ (×), 1α,25(OH)₂D₃ (■), or oil (▲). Arrows indicate the administration of D₃ metabolites. From day 0 to day 20, values are the means ± S.E. of 8 to 20 eggshell weights laid on the indicated days. From day 21 to day 25, each mark represents the individual eggshell weight laid on the indicated day.
tent of medullary bone of the laying quails are shown in Table I. After a 3-week vitamin D-depletion period, medullary bone calcium was markedly reduced from 202.0 ± 29.4 to 61.6 ± 7.1 μg/mg, dry weight, and bone phosphorus from 119.0 ± 15.0 to 33.8 ± 4.2 μg/mg, dry weight. As expected, daily treatment with 1.95 nmol of 25(OH)D3 or 1α,25(OH)2D3 for 4 days resulted in a significant increase in the ash, calcium, and phosphorus content of medullary bone. Administration of 1.95 nmol of 24R,25(OH)2D3 also tended to increase bone ash content slightly, but the same dose of 25(OH)24-oxo-D3 did not. In egg-laying birds, plasma calcium levels rise from normal levels in nonlaying birds to about 20 mg/100 ml for eggshell formation. In this study, the plasma calcium levels of egg-laying quails decreased from 20.0 ± 2.0 to 13.0 ± 1.7 mg/100 ml after a 3-week vitamin D depletion period. The low levels of plasma calcium were restored similarly by the treatment with 25(OH)24-oxo-D3, 24R,25(OH)2D3, 25(OH)D3, or 1α,25(OH)2D3 for 4 days. It seems therefore that the hypercalcemic action of the metabolites of vitamin D3 is not related to their effect on the accumulation of calcium in medullary bone.

Metabolism of 25(OH)24-oxo-D3—When kidney homogenates from vitamin D-deficient chicks were incubated with 25(OH)24-oxo[3H]D3, 2 radioactive peaks other than 25(OH)24-oxo[3H]D3 were detected on the Sephadex LH-20 column. One was eluted in the 24,25(OH)2D3 fraction and another was in the 1α,25(OH)2D3 fraction (Fig. 8). When kidney homogenates from chicks supplemented with vitamin D3 were similarly incubated with 25(OH)24-oxo[3H]D3, the radioactivity remaining in the 25(OH)24-oxo-D3 fraction was reduced to one-fifth. Concomitantly, an unknown radioactive peak appeared in the region of fractions 20-25. No radioactivity occurred in the 1α,25(OH)2D3 fraction (Fig. 8). It should be noted that only 50% of the radioactivity was recovered in the chloroform phase of the extracts of kidney homogenates from vitamin D-supplemented chicks, while the recovery from vitamin D-deficient chicks was 87%.

The pooled 24,25(OH)2D3 fraction from vitamin D-deficient and supplemented chicks on the Sephadex columns were
separated into 3 radioactive peaks on high pressure liquid chromatography. These peaks are referred to as peaks I, II, and III. Vitamin D-supplemented chicks produced peaks I and II, while vitamin D-deficient birds gave peaks II and III (Fig. 9). Peak II migrated to exactly the same position as authentic 24R,25(OH)₂D₃ and peak III was eluted in a region between authentic 24R,25(OH)₂D₃ and 1α,25(OH)₂D₃.

The radioactivity in the suspected 1α,25,25(OH)₃D₃ fraction from vitamin D-deficient chicks on the Sephadex column comigrated to exactly the same position as authentic 1α,24R,25(OH)₃D₃ on high pressure liquid chromatography (data not shown). The radioactive peak in the 25(OH)24-oxo-D₃ fraction on the Sephadex column was homogeneous and migrated to exactly the same position as 25(OH)24-oxo-D₃ chemically synthesized on high pressure liquid chromatography (data not shown).

**DISCUSSION**

Chemical synthesis of 25(OH)24-oxo-D₃ has been accomplished. Comparison of the UV, mass, IR, and ¹H NMR spectra of the synthetic 25(OH)24-oxo-D₃ with those of the isolated metabolite established the correctness of the assigned structure.

Chemical synthesis made it possible to examine the biological significance of 25(OH)24-oxo-D₃. The biological activity of 25(OH)24-oxo-D₃ was about equivalent to 24R,25(OH)₂D₃ in stimulating intestinal calcium transport and bone calcium mobilization in vitamin D-deficient chicks. Although, 25(OH)24-oxo-D₃ was less active than 24R,25(OH)₂D₃ at the dose level of 1.95 nmol, the time course study after administration of 6.5 nmol of 25(OH)24-oxo-D₃ revealed that the oxo compound exhibited biological activity similar to 24R,25(OH)₂D₃ in intestine and bone (Fig. 5).

Recently attention has been focused on the specific action of 24R,25(OH)₂D₃ in bone formation. We compared the effect of various metabolites of vitamin D₃ on the recovery of the impaired eggshells and medullary bone formation of egg-laying quails fed a vitamin D-deficient diet. This system appears to be useful for evaluating the effectiveness of vitamin D₃ metabolites in stimulating bone formation, since calcification of medullary bone is sensitive to vitamin D administration and occurs very rapidly. After 4-days administration of either 1α,25(OH)₂D₃ or 25(OH)D₃, the impaired eggshell weights were restored completely to normal, while 24R,25(OH)₂D₃ and 25(OH)24-oxo-D₃ only partially restored the weights (Fig. 7). All of the metabolites of vitamin D₃ examined were similarly capable of increasing serum calcium concentrations. However, the most effective metabolite of vitamin D₃ in increasing the ash content of medullary bone was 25(OH)D₃, followed successively by 1α,25(OH)₂D₃ and 24R,25(OH)₂D₃. 25(OH)24-oxo-D₃ failed to stimulate calcification of medullary bone, although the oxo compound apparently showed hypercalcemic action similar to that of 25(OH)D₃ (Table I). It is interesting that 25(OH)D₃ was more potent than 1α,25-(OH)₂D₃ in stimulating calcification of medullary bone. 25(OH)D₃ administered to laying quails fed a vitamin D-deficient diet was probably converted into 1α,25(OH)₂D₃ gradually and the plasma levels of 1α,25(OH)₂D₃ must have been maintained within a normal range during the experimental period. Plasma levels of 1α,25(OH)₂D₃ of the quails receiving 1α,25(OH)₂D₃ on the other hand, would have decreased before the next administration of 1α,25(OH)₂D₃. This seems to be one of the reasons why 1α,25(OH)₂D₃ did not show significant effects on calcification of medullary bone. An alternate explanation is the possibility that a synergistic effect of 1α,25(OH)₂D₃ and 24,25(OH)₂D₃ may be required for bone formation. This possibility needs further investigation.

When 25(OH)24-oxo[¹H]D₃ was incubated with kidney homogenates from vitamin D-deficient chicks, a radioactive peak at the dose level of 1.95 mol, the time course study after administration of 6.5 nmol of 25(OH)24-oxo-D₃ revealed that the oxo compound is reduced to 24,25(OH)₂D₃ in part irrespective of the vitamin D status. It has been established that 24,25(OH)₂D₃ is produced only in vitamin D-supplemented animals (5, 27). Plasma levels of 24,25(OH)₂D₃ increase in parallel with the increase of plasma 25(OH)D₃ levels (28). Similarly, 25(OH)24-oxo-D₃ is not produced in the kidney of vitamin D-deficient chicks, and the enzyme responsible for the production of 25(OH)24-oxo-D₃ appears to be induced by 1α,25(OH)₂D₃ (29). In addition, we have found that the production of 25(OH)24-oxo-D₃ increases in parallel with the increase of the amounts of 25(OH)D₃ added as substrate (29). Radioactivity eluted in the 25(OH)24-oxo-D₃ fraction from chicks supplemented with vitamin D₃ was only one-fifth of that from vitamin D-deficient chicks. Moreover, the radioactivity recovered in the chloroform phase decreased significantly in the incubates from chicks supplemented with vitamin D₃ compared with those from vitamin D-deficient chicks. Thus it is conceivable that 25(OH)24-oxo-D₃ synthesized from 24,25(OH)₂D₃ is converted to water-soluble compounds such as the 25,26,27-trinor-D₃-24-carboxylic acid reported by DeLuca and Schoues (15).

These results suggest that the 24-oxidation of 25(OH)D₃ may be a route of inactivation of vitamin D₃. 25(OH)₂D₃ may be converted to either 1α,25-dihydroxy-24-oxovitamin D₃ or 1α,24,25(OH)₃D₃ in vitamin D-deficient chicks before exerting biological activity in bone and intestine. It seems possible that peak III in Fig. 9 may be 1α,25(OH)₂24-oxo-D₃, since peak III appeared only in the incubates from vitamin D-deficient chicks. 25(OH)24-oxo-D₃ was slightly less similar to 25(OH)24-oxo-D₃ than those of authentic 24R,25(OH)₂D₃, and 25(OH)24-oxo-D₃ was less active than 24R,25(OH)₂D₃ at the dose level of 1.95 mol, the time course study after administration of 6.5 nmol of 25(OH)24-oxo-D₃ revealed that the oxo compound exhibited biological activity similar to 24R,25(OH)₂D₃ in intestine and bone (Fig. 5).

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