Role of kidney tissue in the production of 25-hydroxyvitamin D₃-26,23-lactone and 1α,25-dihydroxyvitamin D₃-26,23-lactone

(vitamin D/kidney/25-hydroxyvitamin D/1,25-dihydroxyvitamin D)

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ABSTRACT   Labeled 25-hydroxyvitamin D₃-26,23-lactone was isolated from the serum of vitamin D-repleted rats given [3α-³H]-25-hydroxyvitamin D₃ 24 hr prior to sacrifice. The metabolite was identified by cochromatography with the authentic lactone on straight-phase and reversed-phase high-performance liquid chromatography. Production of the lactone was abolished by nephrectomy indicating that the kidney is the site of synthesis. Homogenate of kidneys from chickens given large doses of vitamin D can carry out *in vitro* production of the lactone from 25-hydroxyvitamin D₃. When 1α,25-dihydroxyvitamin D₃ was used as substrate, this system produced only traces of a compound believed to be 1α,25-dihydroxyvitamin D₃-26,23-lactone. However, incubation of rachitic chicken kidney homogenates with 25-hydroxyvitamin D₃-26,23-lactone produced substantial amounts of a compound that has been identified by mass spectrometry as 1α,25-dihydroxyvitamin D₃-26,23-lactone. Thus, the development of a functional group on C-26 and eventual lactone formation takes place in kidney by a system acting on 25-hydroxyvitamin D₃.

Since the discovery that 25-hydroxyvitamin D₃ (25-OH-D₃) is the major circulating form of vitamin D₃ (1, 2), the further metabolism of vitamin D has been the subject of intensive investigation. Some of the metabolites have been structurally identified and their physiological functions are well defined, but others either remain unidentified or their functions are unknown (1, 2). The 25-hydroxylation of vitamin D₃ takes place in liver; the further hydroxylation of 25-OH-D₃ to 1α,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃] (3), 24,25-dihydroxyvitamin D₃ [24,25-(OH)₂D₃] (4–6), and 25,26-dihydroxyvitamin D₃ [25,26-(OH)₂D₃] (7) takes place in kidney and elsewhere. Recently, a new metabolite of the vitamin has been isolated from the plasma of chickens and identified as 25-hydroxyvitamin D₃-26,23-lactone (25-OH-D₃-26,23-lactone) (8). This metabolite was first observed as an interfering substance in the determination of plasma levels of 24,25-(OH)₂D₃ by the use of the competitive rat plasma protein radioassay method (9). We have investigated the site of biosynthesis of 25-OH-D₃-26,23-lactone from 25-OH-D₃ and its 1α-hydroxylated analog. Our results suggest that lactone formation occurs in kidney and that the 25-OH-D₃-26,23-lactone can serve as a substrate for the production by the renal 1α-hydroxylase system of the corresponding 1α-hydroxylactone [1α-dihydroxyvitamin D₃-26,23-lactone; 1,25-(OH)₂D₃-26,23-lactone] analog. While this work was in progress, evidence for the kidney as the site of synthesis of the 25-OH-D₃-26,23-lactone was provided.†

MATERIALS AND METHODS

Vitamin D Metabolites. [3α-³H]-25-OH-D₃, [23,24-³H]-25-OH-D₃ (10), and [26,27-³H]-25-OH-D₃ (11) were synthesized in our laboratories. Crystalline 24R,25-(OH)₂D₃, 1,24R,25-trihydroxyvitamin D₃ [1,24R,25-(OH)₃D₃] and 1,25-(OH)₂D₃ were gifts from Hoffmann–LaRoche. 25,26-(OH)₂D₃ was synthesized by the method of Lam et al. (12). 25-OH-D₃-26,23-lactone was isolated from chicken plasma by the method of Wichmann et al. (8). 25-OH-D₃ was a gift from Upjohn.

Animals. For *in vivo* production of 25-OH-D₃-26,23-lactone, male weanling rats were purchased from Holtzman, Madison, WI, and fed an adequate calcium, adequate phosphorus diet (13) supplemented with 25 international units of vitamin D₃ per day for 6 weeks Two days before the conclusion of the experiment, the rats were given 25-OH-D₃ (5 μg in 0.1 ml of 1,2-propandiol per day) subcutaneously. Twenty hours before sacrifice, the rats were either nephrectomized or sham operated and then given intrajugularly 10 μCi [3α-³H]-25-OH-D₃ (28 Ci/mmol; 1 Ci = 3.7 × 10¹⁰ becquerels) in 0.05 ml of 95% ethanol.

For *in vitro* production of 25-OH-D₃-26,23-lactone and 1α,25-(OH)₂D₃-26,23-lactone, single-comb White Leghorn chickens (Northern Hatcheries, Beaver Dam, WI) were fed a normal diet for 10 weeks and given 10⁵ international units of vitamin D₃/day for 3 days, followed by 1.5 × 10⁷ international units for 1 day, 4 days before sacrifice.

For *in vitro* production of 1α,25-(OH)₂D₃-26,23-lactone from 25-OH-D₃-26,23-lactone, one-day old male White Leghorn chickens (Northern Hatcheries) were fed a rachitogenic diet (14) for 4 weeks.

Extraction of Rat Serum. Twenty-four hours after the administration of [3α-³H]-25-OH-D₃, rats were killed and their blood was collected. The blood was centrifuged to yield serum. Seven milliliters of serum was pooled from three rats in either the nephrectomized or the sham-operated group. The serum was mixed with authentic 1,25-(OH)₂D₃, 25,26-(OH)₂D₃, and

24R,25-dihydroxyvitamin D_3 [24R,25-$(OH)_2D_3$] (200 ng each) and 100 ng of 25-OH-D_3-26,23-lactone dissolved in 50 μl of ethanol. After 1-hr incubation at room temperature, extractions of the sera were carried out (15).

In Vitro **Incubation of Chicken Kidney or Liver Homogenate with Labeled Substrate.** Chickens were killed by decapitation, and their kidneys were placed in ice-cold 0.25 M sucrose. A 20% homogenate of kidney was prepared in 0.25 M sucrose. Liver homogenate was prepared in the same way. Incubation was carried out in a 25-ml erlenmeyer flask that contained, in a 2-ml total volume, 200 mg of tissue, 50 mM phosphate buffer, pH 7.4/22.4 mM glucose 6-phosphate/20 mM ATP/160 mM nicotinamide/0.4 mM NADP/5 mM $MgCl_2$/0.1 M KCl/glucose 6-phosphate dehydrogenase (0.5 units) (Sigma). The reaction was initiated by addition of 0.4 μCi of either [3α-^{3}H]-25-OH-D_3, [23,24-^{3}H]-25-OH-D_3, or [26,27-^{3}H]25-OH-D_3 in 10 μl of 95% ethanol. The mixtures were incubated at 37°C with shaking at 100 oscillations per min for 1 hr under an atmosphere of air. The reaction was stopped by addition of methanol/chloroform (2:1). One hundred nanograms each of authentic 24R,25-$(OH)_2D_3$ and 25-OH-D_3-26,23-lactone was added to each flask. After 1 hr of incubation at room temperature, the incubation mixture was extracted (15).

In Vitro **Incubation of Chicken Kidney Homogenate with Unlabeled 25-OH-D_3 or 1,25-$(OH)_2D_3$.** Incubation conditions were as described above except that incubation was carried out in a 4-ml reaction volume in a 125-ml erlenmeyer flask containing 400 mg of kidney tissue. In some experiments, the glucose 6-phosphate and glucose 6-phosphate dehydrogenase was replaced by 25 mM sodium succinate (Table 1). The reaction was initiated by addition of either 400 μg of 25-OH-D_3 or 20 μg of 1,25-$(OH)_2D_3$ in 100 μl of 95% ethanol. The mixtures were incubated for 1 hr at 37°C with shaking at 100 oscillations per min. The reaction was stopped by addition of methanol/chloroform (2:1) and extracted as described (15).

Production of 1,25-$(OH)_2D_3$-26,23-Lactone from 25-OH-D_3-26,23-Lactone. Kidney homogenate (20% wt/vol) from rachitic chickens was prepared as described above in 0.19 M sucrose/15 mM Tris-acetate/1.9 mM $MgCl_2$. Homogenate (600 mg) was incubated (total volume 4.5 ml) in 0.19 M sucrose/15 mM Tris-acetate, pH 7.4/1.9 mM $MgCl_2$/25 mM sodium succinate in a 125-ml erlenmeyer flask using an air atmosphere and shaking at 100 oscillations per min. To initiate the reaction, 50 μl of ethanol containing 10 μg of 25-OH-D_3-26,23-lactone was added. The incubation was continued for 2.5 hr and terminated by the addition of methanol/chloroform (2:1). Extraction was carried out as described (15).

Purification of Extract by Sephadex LH-20 Chromatography. To purify the 25-OH-D_3-26,23-lactone, the lipid extract of either serum or incubation medium was applied to a Sephadex LH-20 column (0.7 × 14 cm) packed in 65% $CHCl_3$/hexane as described (16). The column was eluted with the same

Table 1. Rat plasma protein competitive binding assay of 25-OH-D_3-26,23-lactone produced *in vitro*

Source of reducing equivalents	Lactone produced, ng per vessel
NADPH-generating system	6.8
Succinate	3.4
None	0

Kidney homogenate was incubated with 25-OH-D_3 as described. After purification of the putative lactone by high-performance liquid chromatography (see Fig. 2), the amount of 25-OH-D_3-26,23-lactone produced *in vitro* was determined.

solvent; the first 11 ml of eluant was discarded, and the next 25 ml was collected. The elution positions of the metabolites were established with the authentic compounds. The initial purification of 1,25-$(OH)_2D_3$-26,23-lactone produced by chicken kidney homogenates involved chromatography of the extracts on Sephadex LH-20 (1 × 16 cm column) and elution with $CHCl_3$/hexane/MeOH (975:23:2). The first 45 ml of eluant was discarded, and the next 80 ml [representing the elution volume of 1,24R,25-$(OH)_3D_3$ used as calibration standard] was pooled for subsequent high-performance liquid chromatography (HPLC) purification.

HPLC of Rat Serum Extract. The purified extract of serum from either nephrectomized or sham-operated rats was dried under nitrogen, dissolved in 8% 2-PrOH/hexane and subjected to HPLC using a Zorbax-Sil column (4.6 mm × 25 cm). The HPLC was performed at a pressure of 1000 lbf/in² (1 lbf/in² = 6895 Pa) and a flow rate of 2 ml/min; the solvent was 8% 2-PrOH/hexane. Fractions of 0.8 ml were collected, and aliquots were counted in toluene counting solution (17). UV absorbance was monitored at 254 nm by a model 440 absorbance detector (data not shown). The fractions containing 25-OH-D_3-26,23-lactone (16–18 ml) were combined and applied to the reversed-phase HPLC using a Lichrosorb RP-18 column (4.6 mm × 25 cm) (E. Merk, Darmstadt, West Germany); this was eluted with 22% H_2O/MeOH at a pressure of 1300 lbf/in² and a flow rate of 2 ml/min (Fig. 1).

HPLC of Extracts from Incubations. To further purify the 25-OH-D_3-26,23-lactone, the extracts were treated as described above, except that the straight-phase HPLC was eluted with 6% 2-PrOH/hexane (Fig. 2A). Fractions of 0.8 ml were collected, and an aliquot of each fraction was counted as above. The fractions containing 25-OH-D_3-26,23-lactone (Fig. 2A) were combined and applied to the reversed-phase HPLC as described above except that the elution solvent was 25% H_2O/MeOH. Fractions of 0.8 ml each were collected and counted (Fig. 2B).

The extracts of tissue incubated with unlabeled 25-OH-D_3 were subjected to straight-phase HPLC as described above, and the 25-$(OH)_2D_3$-26,23-lactone fraction (16–19 ml) was collected. The putative 25-OH-D_3-26,23-lactone was then subjected to reversed-phase HPLC as described above, and the lactone fraction (22–27 ml) was collected.

For the experiments dealing with 1,25-$(OH)_2D_3$-26,23-lac-

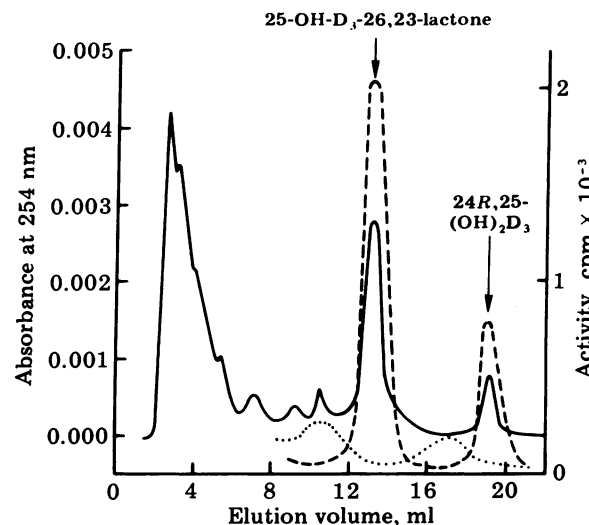FIG. 1. HPLC profiles of extracts of serum from sham-operated (- - -) or nephrectomized (·····) rats given [3α-^{3}H]-25-OH-D_3. (——) UV absorbance at 254 nm; (- - -) and (·····), radioactivity. Arrows indicate elution positions of authentic compounds.
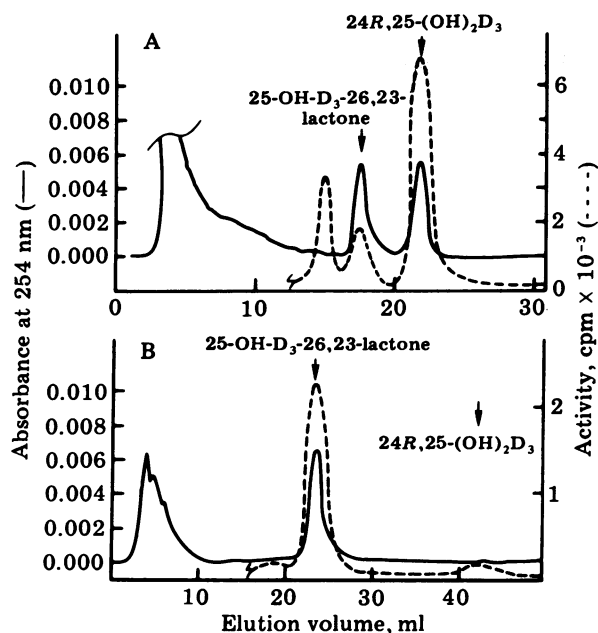
FIG. 2. HPLC profiles of extracts of kidney homogenate from chickens given high doses of vitamin D and incubated with [3α-³H]-25-OH-D₃. (*A*) Zorbax-Sil column was eluted with 6% 2-PrOH/ hexane at a pressure of 1000 lbf/in² and a flow rate of 2 ml/min. (*B*) Reversed-phase HPLC of 25-OH-D₃-26,23-lactone fraction from *A*. The fractions (16–19 ml) from straight-phase HPLC (*A*) were dried, dissolved in 25% H₂O/MeOH and subjected to HPLC on a Lichrosorb RP-18 column. HPLC was performed with 25% H₂O/MeOH at 1300 lbf/in² and a flow rate of 2 ml/min.

tone, the extracts were subjected to straight-phase HPLC using 15% 2-PrOH/hexane after prepurification on Sephadex LH-20. The putative 1,25-(OH)₂D₃-26,23-lactone from the two substrates used was collected and rechromatographed on a reversed-phase system (Zorbax-ODS) using 35% H₂O/MeOH as eluting solvent (Fig. 3). The product eluting at 17 ml was collected and applied to a straight-phase silica gel column (Zorbax-Sil); this was eluted with 15% 2-PrOH/hexane. The resulting product (eluting at 29 ml) was then subjected to physical characterization.

Rat Plasma Protein Competitive Binding Assay. The fractions containing 25-OH-D₃-26,23-lactone collected from the reversed phase HPLC were dried under N₂ and dissolved in 100 μl of ethanol. Duplicate 50 μl portions of each were used for measurement of 25-OH-D₃-26,23-lactone by a rat plasma protein competitive assay (9).

General Procedures. All HPLC was carried out on a Waters Associates ALC/GPC 204 instrument equipped with either a fixed-wavelength UV detector (at 254 nm) or a variable-wavelength detector (at 265 nm). Scintillation counting was performed by using a Packard Instruments (Downers Grove, IL) 3255 spectrometer and mass spectrometry was performed by using an AEI MS-9 instrument (Associated Electrical Industries, Manchester, England) having a direct probe inlet and operated at 70 eV and a source temperature of 140°C above ambient.

RESULTS

When vitamin D repleted rats with intact kidneys were given [3α-³H]-25-OH-D₃ the main metabolite observed was 24,25-(OH)₂D₃ with much less 25,26-(OH)₂D₃, 1,25-(OH)₃D₃, and a substance that comigrated with authentic 25-OH-D₃-26,23-lactone. In contrast, nephrectomized rats produced almost none of these metabolites. The fraction containing the putative
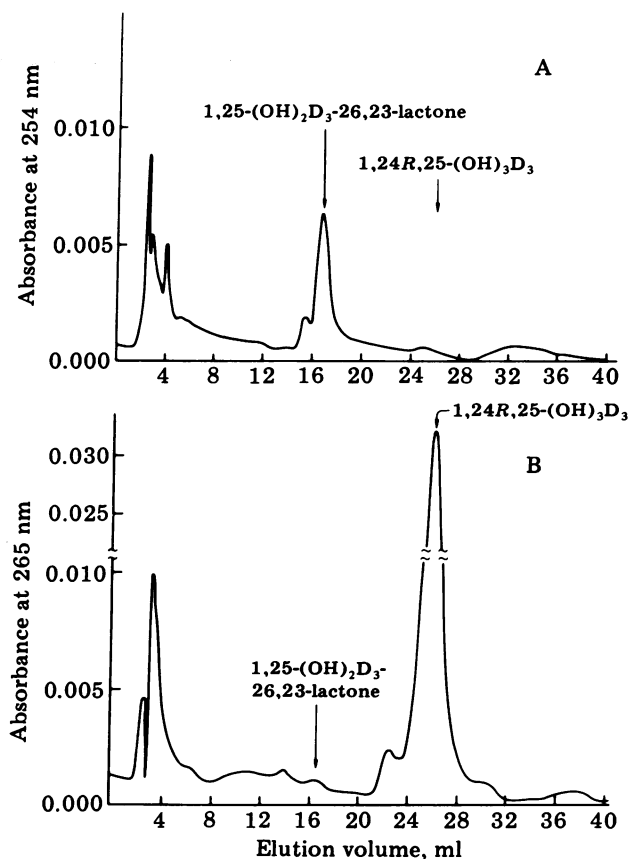

FIG. 3. HPLC profiles of extracts of rachitic chicken kidney homogenate incubated with 25-OH-D₃-26,23-lactone (*A*) and kidney homogenate taken from chickens given large doses of vitamin D incubated with 1,25-(OH)₂D₃ (*B*). System used was a Zorbax-Sil column developed with 15% 2-PrOH/hexane. The 1,25-(OH)₂D₃-26,23-lactone from *A* was identified by mass spectrometry.

25-OH-D₃-26,23-lactone was further purified by using reversed-phase HPLC (see Fig. 1). The radioactive substance from the serum of sham-operated rats comigrated with authentic 25-OH-D₃-26,23-lactone on this system. Furthermore, the metabolite was totally absent in nephrectomized rats (Fig. 1).

Kidney homogenate from vitamin D-treated chickens produced 25-OH-D₃-26,23-lactone from [3α-³H]-25-OH-D₃ (see Fig. 2). The lactone fraction from straight-phase HPLC was then subjected to reversed-phase HPLC; the radioactive peak comigrated with authentic 25-OH-D₃-26,23-lactone. Liver homogenate does not produce lactone from [3α-³H]-25-OH-D₃ (data not shown). Further, no lactone is produced by kidney homogenate incubated with 23,24-³H- or 26,27-³H-labeled 25-OH-D₃, presumably because of isotope effect (data not shown).

The amount of 25-OH-D₃-26,23-lactone produced by incubation of kidney homogenates with unlabeled 25-OH-D₃ was not detectable by UV absorbance but was detectable by rat serum protein competitive binding assay.

When 25-OH-D₃-26,23-lactone was incubated with kidney homogenates prepared from rachitic chickens, a product was formed that eluted just prior to 1,24R,25-(OH)₃D₃ on HPLC (see Fig. 3). By a combination of HPLC steps, sufficient material was isolated for physical characterization. The mass spectrum of the compound showed a molecular ion at m/e = 444, as expected for the 1-hydroxylated form of 25-OH-D₃-26,23-lactone, and peaks due to elimination of H₂O and methyl radical at m/e = 426 (M⁺ − H₂O), 411 (M⁺ − H₂O − CH₃),

and 408 ($M^+ - 2 \times H_2O$). In addition, the spectrum showed characteristic fragment peaks at $m/e = 269$ (M^+ − sidechain − H_2O) and 251 (269 − H_2O), as well as the ring A fragments at $m/e = 152$ and 134 diagnostic for 1-hydroxylated vitamin D derivatives. The characteristic intensity ratio of the peaks at $m/e = 152$ and 134 (40:100) further established the α-configuration for the 1-hydroxy function, because it is known (18) that a 1β-hydroxy substituent leads to a 152:134 peak ratio of 100:90. Given the formation of this metabolite from 25-OH-D_3-26,23-lactone, these data prove the compound to be 1,25-$(OH)_2D_3$-26,23-lactone.

In view of this result, it was of interest to determine whether chicken kidney preparations capable of converting 25-OH-D_3 to its lactone could also use $1\alpha,25$-$(OH)_2D_3$ as substrate for conversion to the 1α-hydroxylactone analog. Incubation of $1\alpha,25$-$(OH)_2D_3$ with kidney homogenates from chickens given large doses of vitamin D_3 gave (as expected) a product that was chromatographically identical (on two HPLC systems) with 1,24,25-$(OH)_3D_3$, but only a trace of material corresponding in elution position to $1\alpha,25$-$(OH)_2D_3$-26,23-lactone.

DISCUSSION

The isolation and characterization of 25-OH-D_3-26,23-lactone (8) and the fact that this compound is a major vitamin D metabolite under conditions of high intake raise questions concerning its possible *in vivo* function, mode of biosynthesis, and site of production.

The involvement of kidney in lactone formation is evident from initial *in vivo* experiments with rats on a normal vitamin D diet. The results show that nephrectomy prevents the conversion of 25-OH-D_3 to the lactone, whereas sham animals carry out the conversion. As anticipated, the production of 1,25-$(OH)_2D_3$ and of 24,25-$(OH)_2D_3$ was either eliminated or greatly reduced by nephrectomy (19, 20). These results, therefore, suggest that the kidney plays an important role in production of the lactone. Kidney homogenates from chickens given high doses of vitamin D readily produced a compound that comigrated on straight-phase HPLC with authentic 25-OH-D_3-26,23-lactone. The putative lactone also comigrated with authentic lactone on reversed-phase HPLC, a system known to be a very effective method for the resolution of the lactone and 24,25-$(OH)_2D_3$ compounds (8). It is, therefore, evident from both *in vivo* and *in vitro* experiments that production of the 25-OH-D_3-26,23-lactone takes place largely in the kidney (although biogenesis in other tissues cannot be excluded at this time).

Because the kidney can produce the lactone from 25-OH-D_3, it was of interest to determine whether that organ could also produce a lactone from 1,25-$(OH)_2D_3$. The results show that kidney preparations from chickens given large doses of vitamin D readily convert 1,25-$(OH)_2D_3$ to 1,24,25-$(OH)_3D_3$ but produce only trace amounts of 1,25-$(OH)_2D_3$-26,23-lactone. Evidently 1,25-$(OH)_2D_3$ is a poor substrate analog for the system effecting the conversion of 25-OH-D_3 to the lactone. 1,25-$(OH)_2D_3$-26,23-Lactone could be produced, however, by C-1-hydroxylation of 25-OH-D_3-26,23-lactone. Incubation of 25-OH-D_3-26,23-lactone with homogenates obtained from rachitic chickens known to be rich in 1-hydroxylase activity and deficient in other vitamin D hydroxylases (1, 2) converted the lactone to a more polar compound that eluted on HPLC somewhat before 1,24R,25-$(OH)_3D_3$ (Fig. 3A). This compound was isolated and identified as 1,25-$(OH)_2D_3$-26,23-lactone by mass spectrometry. Presumably, 1-hydroxylation of 25-OH-

D_3-26,23-lactone involves the enzyme that normally catalyzes the conversion of 25-OH-D_3 to 1,25-$(OH)_2D_3$. That system appears specific for a 25-hydroxy group but can accommodate other side-chain modifications in the substrate, as indicated, for example, by the conversion of both 24,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$ to the corresponding 1α-hydroxy analogs by chicken kidney 1α-hydroxylase. Because 1α-hydroxylase activity is generally low under the conditions in which the lactone is formed (9), it is unlikely that much 1,25-$(OH)_2D_3$-26,23-lactone can be formed by 1-hydroxylation *in vivo*, and the small amounts of 1,25-$(OH)_2D_3$-26,23-lactone formed from 1,25-$(OH)_2D_3$ *in vitro* by the lactonizing system suggests that this pathway is also minimal *in vivo*. In support of this, only small amounts of 1,25-$(OH)_2D_3$-26,23-lactone (15 ng/6 liters) could be detected in the plasma of chickens containing substantial amounts of 25-OH-D_3-26,23-lactone (200 μg/6 liters).

The biological importance of 25-OH-D_2-26,23-lactone and its 1α-hydroxy analog remains unknown. The 25-OH-D_3-26,23-lactone has a weak effect on intestinal calcium transport and bone calcium mobilization, and it is likely that the effect of the 1α-hydroxylated compound will be similar. However, it is possible that these compounds have an important role in other aspects of vitamin D action, a possibility that can be ascertained only by further investigation.

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