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25,26-DIHYDROXYCHOLECALCIFEROL: A PRECURSOR IN THE RENAL SYNTHESIS OF 25-HYDROXYCHOLECALCIFEROL-26,23-LACTONE

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SUMMARY

Both rachitic 1,25-dihydroxycholecalciferol-treated and normal chicks produced detectable quantities of 25-hydroxycholecalciferol-26,23-lactone, although renal homogenates from rachitic chicks failed to produce this compound. The addition of either 25-hydroxycholecalciferol or 25,26-dihydroxycholecalcifero to the renal homogenates led to the production of the lactone, although more lactone resulted when 25,26-dihydroxycholecalciferol was used. When tritiated 25-hydroxycholecalciferol was added to rachitic 1,25-dihydroxycholecalciferol-treated or normal chick renal homogenates, an unidentified tritiated vitamin D metabolite distinct from 25-hydroxycholecalciferol-26,23-lactone was produced.

INTRODUCTION

A recently isolated vitamin D_3 metabolite, 25-hydroxycholecalciferol-26,23-lactone[1], has been shown to compete actively for the vitamin D binding protein in the ligand binding assay for 25-OH-D_3 (1). Apparently this lactone is produced in the kidney (1), although its physiological activity, precursor(s), and regulation of synthesis have not been explained. Our investigation of the lactone focused on whether a known vitamin D metabolite is a precursor in the lactone's renal synthesis.

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[1] ABBREVIATIONS USED

25-OH-D_3: 25-hydroxycholecalciferol
24,25-$(OH)_2$-D_3: 24,25-dihydroxycholecalciferol
25,26-$(OH)_2$-D_3: 25,26-dihydroxycholecalciferol
1,25-$(OH)_2$-D_3: 1,25-dihydroxycholecalciferol
1,24,25-$(OH)_3$-D_3: 1,24,25-trihydroxycholecalciferol
Calcidiol lactone: 25-hydroxycholecalciferol-26,23-lactone
HPLC: high performance liquid chromatography
IU: international unit

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Materials and Methods

Sterols. Crystalline 25-OH-D_3 was contributed by The Upjohn Company (Kalamazoo, MI). Crystalline 24R,25-$(OH)_2$-D_3 and 1,25-$(OH)_2$-D_3 were gifts from Hoffman-LaRoche (Nutley, NJ). Standard calcidiol lactone and 25,26-$(OH)_2$-D_3 were produced in vivo by the method of Horst (8). The calcidiol lactone and 25,26-$(OH)_2$-D_3 were identified by their chromatographic mobilites on a number of different HPLC systems in conjunction with direct probe mass spectrometry. Diagnostic ions from the mass spectral analysis of calcidiol lactone (m/e 118, 136, 253, 271, 295, 410, and 428) and 25,26-$(OH)_2$-D_3 (m/e 118, 136, 253, 271, 383, and 416) agree with previous reports (1-3)[2]. The biosynthetic sterols demonstrated the 5,6-cis-triene ultraviolet spectrum (absorption maximum at 265 nm and minimum at 228 nm) characteristic of vitamin D_3 and its metabolites (4). The 25-OH-[26,27-^{3}H]-D_3 (8 Ci/mmole), 24,25-$(OH)_2$-[23,24-^{3}H]-D_3 (94 Ci/mmole), and 1,25-$(OH)_2$-[23,24-^{3}H]-D_3 (110 Ci/mmole) were purchased from Amersham Searle (Chicago, IL). The 25,26-$(OH)_2$-[26,27-^{3}H]-D_3 (8 Ci/mmole) was synthesized according to the method of Tanaka et al. (5).

Animals. One-day-old leghorn chicks were obtained from Kirby Hatcheries (Urbana, OH) and divided into two groups. One group of chicks was fed a vitamin D-deficient diet (Nutritional Biochemicals Inc., Cleveland, OH) while the other group received a normal chick diet containing 1,000 IU/kg feed of vitamin D_3 for a period of 4 weeks. Some of the vitamin D deficient chicks were given four subcutaneous injections of 1,25-$(OH)_2$-D_3 (500 ng/injection) in 0.05 ml ethanol over a three day period before they were killed by decapitation for the preparation of renal homogenates.

In vitro incubation of kidney homogenates. Three groups of chicks were utilized for kidney incubations. These groups (4 chicks/group) consisted of normal, rachitic, or 1,25-$(OH)_2$-D_3-injected rachitic chicks. The kidneys from each group were pooled and a 20% (w/v) homogenate was prepared in ice-cold 0.20 M sucrose containing 15 mM Tris acetate (pH 7.4), 2.0 mM magnesium chloride, and 25 mM succinate. The incubations were carried out in duplicate in 25-ml Erlenmeyer flasks which contained 2 ml of the appropriate homogenate. Each homogenate was aliquotted and aliquots incubated with one of the following substrates: ^{3}H-25-OH-D_3 (2 µCi), 25-OH-D_3 (250 ng), 24,25-$(OH)_2$-D_3 (250 ng), or 25,26-$(OH)_2$-D_3 (250 ng). One aliquot of each homogenate served as a control (i.e. no added substrate). All of the above substrates were added to the appropriate flask in 0.05 ml ethanol and each flask was gassed with 100% O_2 for 30 sec before the incubation at 37°C for 1.5 hrs. The reactions were stopped by the addition of 3 volumes (v/v) of a CH_3OH:CH_2Cl_2 (2:1, v/v) to each flask. The extraction of the metabolites was performed as previously described (6). The extracts were then dried under N_2 and stored at -70°C in absolute ethanol.

Column chromatography. Extracts of the ^{3}H-25-OH-D_3 incubations were applied to a column of Sephadex LH-20 (0.9 x 22 cm) developed and eluted with $CHCl_3$:n-hexane (65:35, v/v) (7). Extracts from the remaining incubations were applied to a column of Sephadex LH-20 (0.9 x 17 cm) developed and eluted with n-hexane:$CHCl_3$:CH_3OH (9:1:1, v/v) to resolve 25-OH-D_3 from the remaining more polar metabolites (6). The pooled chromatographic peaks from Sephadex LH-20 were further purified by normal phase HPLC using a Waters Model 202 high performance liquid chromatograph equipped with a µ-porasil column. Solvent systems of n-hexane:isopropanol (92:8, or 97/3, v/v) with flow rates of 2.0 ml/min were used for the separation of the various vitamin D metabolites. Competitive protein binding assays of various HPLC chromatographic fractions were performed as previously described (1,8-9).

[2]Mass spectral data for calcidiol lactone and 25,26-$(OH)_2$-D_3 will be made available upon request to the authors.

RESULTS

Our chromatographic system resolved the generated ^{3}H-vitamin D metabolites into 4 groups which included fractions that corresponded to: (1) pre-25-OH-D_3; (2) 25-OH-D_3; (3) 24,25-$(OH)_2$-D_3 and calcidiol lactone; and (4) 25,26-$(OH)_2$-D_3 and 1,25-$(OH)_2$-D_3. The elution profile of these metabolites on this chromatographic system agrees with previous reports (1,3). These fractions were further resolved by normal phase HPLC. Resolution of the fraction containing 25,26-$(OH)_2$-D_3 and 1,25-$(OH)_2$-D_3 was achieved on a normal phase HPLC system eluted with n-hexane:isopropanol (92:8, v/v). While this HPLC system was adequate for the separation of 25,26-$(OH)_2$-D_3 from 1,25-$(OH)_2$-D_3, with retention times of 9 min and 15 min, respectively, it was not sufficient for the separation of 24,25-$(OH)_2$-D_3 from calcidiol lactone. The fraction containing 24,25-$(OH)_2$-D_3 and calcidiol lactone was applied to a less polar HPLC system. This HPLC system was eluted with n-hexane:isopropanol (97:3, v/v) which gave retention times of 14 min and 20 min, respectively, for these two metabolites.

Results of the renal incubations from the various chick groups incubated with ^{3}H-25-OH-D_3 and subjected to the appropriate chromatographic procedures yielded the following. Renal homogenates from rachitic chicks metabolized ^{3}H-25-OH-D_3 to peak X(0.3%), 24,25-$(OH)_2$-D_3 (2.5%), 25,26-$(OH)_2$-D_3 (1.7%) and 1,25-$(OH)_2$-D_3 (95.5%). ^{3}H-25-OH-D_3 was metabolized in 1,25-$(OH)_2$-D_3-injected chick renal homogenates to give peak X (32.0%), 24,25-$(OH)_2$-D_3 (46.0%), 25,26-$(OH)_2$-D_3 (19.0%) and 1,25-$(OH)_2$-D_3 (3.0%). Finally, renal homogenates from normal chicks metabolized ^{3}H-25-OH-D_3 to peak X (20.0%), 24,25-$(OH)_2$-D_3 (77.0%), 25,26-$(OH)_2$-D_3 (2.0%) and 1,25-$(OH)_2$-D_3 (1.0%). The absolute conversion of ^{3}H-25-$(OH)_2$-D_3 to other metabolites in the renal homogenates for the rachitic, 1,25-$(OH)_2$-D_3-treated and normal chicks was 79%, 45% and 47%, respectively. The data confirm earlier reports that the vast majority of the more polar metabolites isolated from the rachitic chick renal homogenates consist of ^{3}H-1,25-$(OH)_2$-D_3 with only minor amounts of

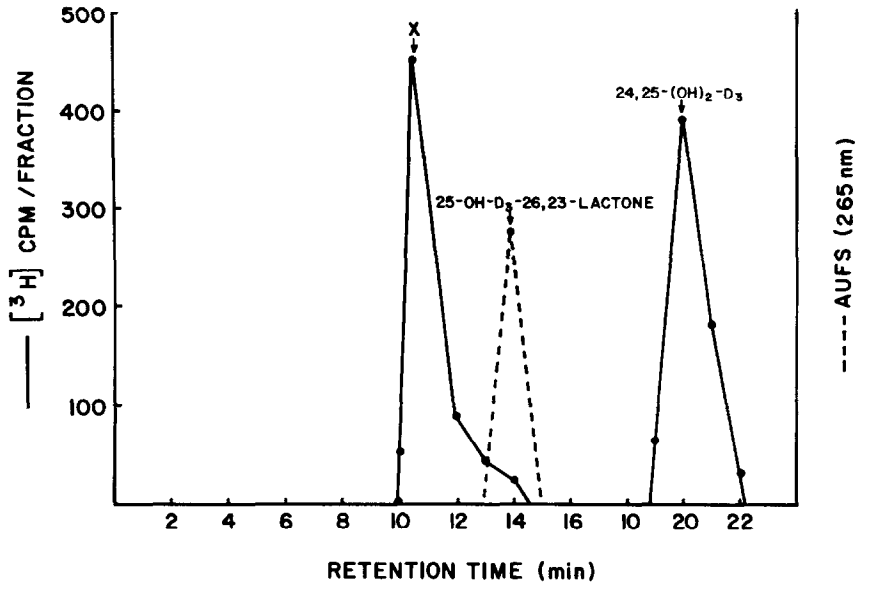

Fig 1. Elution profile of the Sephadex LH-20 [$CHCl_3$:n-hexane (65:35,v/v)] ^{3}H-24,25-$(OH)_2$-D_3 peak from the renal homogenate of chicks treated with 1,25-$(OH)_2$-D_3 prior to sacrifice on normal phase HPLC [n-hexane:isopropanol, (97:3, v/v)]. The elution of authentic calcidiol lactone is depicted by ultraviolet scanning. One-min fractions were collected from the HPLC and monitored for radioactivity.

the other more polar metabolites present (10). These data also demonstrate that chicks injected with 1,25-$(OH)_2$-D_3 prior to sacrifice produce a variety of more polar vitamin D metabolites, the most prominent one being ^{3}H-24,25-$(OH)_2$-D_3. This group of animals also produced significant amounts of ^{3}H-25,26-$(OH)_2$-D_3 and another ^{3}H-vitamin D metabolite that when chromatographed on a less polar HPLC system migrated very closely but distinctly from authentic calcidiol lactone (Figure 1). The normal chick renal homogenates also demonstrated this unknown peak along with a significant production of ^{3}H-24,25-$(OH)_2$-D_3, but they produced very little ^{3}H-25,26-$(OH)_2$-D_3 or ^{3}H-1,25-$(OH)_2$-D_3.

Table 1 illustrates the production of calcidiol lactone using different vitamin D metabolites as substrates in the renal homogenate incubations. Calcidiol lactone was 3.5 times more potent in displacing ^{3}H-25-OH-D_3 from the vitamin D-binding globulin than were the other metabolites tested, confirming previous observations (1). The renal homogenates from

Table 1. The generation of calcidiol lactone from renal homogenates incubated with various vitamin D metabolites.

Chick group[a]	Metabolite added[b]	HPLC fraction[c]	Calcidiol lactone (ng)[d]
Rachitic	25-OH-D_3	1	N.D.[e]
		2	N.D.
	24,25-$(OH)_2$-D_3	1	N.D.
		2	N.D.
	25,26-$(OH)_2$-D_3	1	N.D.
		2	N.D.
1,25-$(OH)_2$-D_3 injected	25-OH-D_3	1	N.D.
		2	0.4
	24,25-$(OH)_2$-D_3	1	N.D.
		2	N.D.
	25,26-$(OH)_2$-D_3	1	N.D.
		2	2.0
Normal	25-OH-D_3	1	N.D.
		2	N.D.
	24,25-$(OH)_2$-D_3	1	N.D.
		2	N.D.
	25,26-$(OH)_2$-D_3	1	N.D.
		2	12.0

[a] The renal homogenate for each chick group was prepared from the kidneys pooled from 4 chicks.

[b] 250 ng of a specific vitamin D metabolite was incubated for 1.5 hr at 37° in 2 ml of a renal homogenate prepared from the designated chick group.

[c] HPLC fractions 1 and 2 correspond to 10-12 and 13-15 minutes retention time, respectively. Fraction 1 would contain the unidentified compound while Fraction 2 would contain calcidiol lactone (Fig 1).

[d] The HPLC fractions were assayed for their calcidiol lactone content using the ligand binding assay as described in the Methods.

[e] Not detectable (< 0.020 ng).

the rachitic chick group produced no detectable calcidiol lactone regardless of the vitamin D metabolite used as a substrate (Table 1) (Figure 2A). Renal homogenates from chicks previously injected with 1,25-$(OH)_2$-D_3 produced calcidiol lactone from both 25-OH-D_3 and 25,26-$(OH)_2$-D_3, although the amount of calcidiol lactone produced from the added 25,26-$(OH)_2$-D_3 was 5 times greater (Table 1) (Figure 2B). Finally, the renal homogenates from the normal chicks also produced significant amounts of calcidiol lactone, 6 times greater than for the 1,25-$(OH)_2$-D_3 injected chicks, although in this group only the addition of 25,26-$(OH)_2$-D_3 led to the production of calcidiol lactone (Table 1) (Figure 2C).

It is also interesting to note in Table 1 that only in fraction 2, which contained calcidiol lactone (Figure 1), could any assay displacement activity be detected. This would indicate that the peak X compound, which migrates in fraction 1 (Figure 1), generated from ^{3}H-25-OH-D_3 and 25-OH-D_3, has little ability to compete in this ligand binding assay.

DISCUSSION

The discovery of a new vitamin D metabolite, calcidiol lactone, has raised the interesting questions about where and from what this metabolite is derived (1,3). Previous work has suggested that calcidiol lactone formation probably occurs in renal tissue (1) although its precursor remains unknown. The data presented in the present study strongly indicate that the precursor to calcidiol lactone is 25,26-$(OH)_2$-D_3, a vitamin D metabolite whose function is virtually unknown (4).

Calcidiol lactone can be derived from either 25-OH-D_3 or 25,26-$(OH)_2$-D_3 upon their incubation with kidney homogenates from rachitic chicks previously injected with 1,25-$(OH)_2$-D_3, although 25,26-$(OH)_2$-D_3 leads to a 5-fold greater production of this metabolite than does 25-OH-D_3 (Table 1) (Figure 2B). When renal homogenates from normal chicks are used only the inclusion of 25,26-$(OH)_2$-D_3 into the medium results in the formation of calcidiol lactone (Table 1) (Figure 2C). From these data it appears that 25-OH-D_3 can lead to

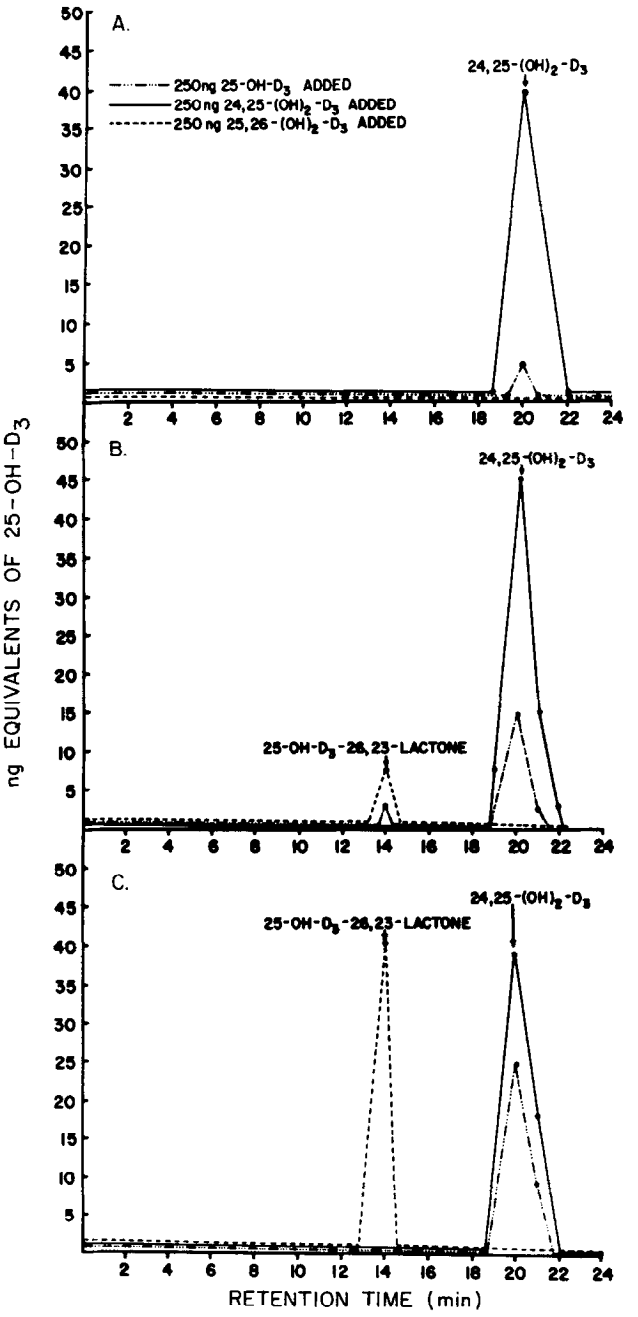

Fig 2. Normal phase HPLC elution profile of the Sephadex LH-20 peak containing 24,25-$(OH)_2$-D_3, calcidiol lactone and 25,26-$(OH)_2$-D_3. These samples were generated from various chick renal homogenates that were incubated with the appropriate vitamin D_3 metabolite. The normal phase HPLC system was eluted with n-hexane:isopropanol (97:3, v/v) with a flow rate of 2 ml/min. One-min fractions were collected from the HPLC and subjected to the ligand binding assay for calcidiol lactone. Panels A, B and C respectively, represent the profile of ligand binding activity for rachitic, 1,25-$(OH)_2$-D_3-treated and normal chick renal homogenates incubated with the vitamin D_3 metabolite noted.

the *in vitro* production of calcidiol lactone but only after first by being converted to 25,26-$(OH)_2$-D_3. Since very little ^{3}H-25,26-$(OH)_2$-D_3 is produced by the renal homogenate from normal chicks it is not surprising that calcidiol lactone could not be detected in the ligand binding assay when 25-OH-D_3 was used as a substrate (Table 1) (Figure 2C).

The data presented demonstrate the presence of a previously unidentified ^{3}H-vitamin D_3 metabolite (Figure 1). The vitamin D metabolite immediately responsible for the production of this compound is unknown although its production appears to be controlled as renal homogenates from rachitic chicks do not produce it while renal homogenates from rachitic chicks previously injected with 1,25-$(OH)_2$-D_3 and from normal chicks produce significant amounts of this compound. This compound is chromatographically distinct from calcidiol lactone and exhibits little, if any, ability to compete in ligand binding assay for 25-OH-D_3 and calcidiol lactone.

The renal enzyme system that controls the proposed formation of calcidiol lactone from 25,26-$(OH)_2$-D_3 appears to be under some type of regulation as rachitic chick renal homogenates fail to produce this compound while renal homogenates from normal or from rachitic 1,25-$(OH)_2$-D_3-injected chicks are able to initiate its synthesis (Table 1) (Figure 2). 25,26-$(OH)_2$-D_3 seems the most likely vitamin D metabolite from which calcidiol lactone could be derived since the hydroxyl function on C26 is available for oxidation and cyclization into a lactone function.

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