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Biological Activity of 1α-Hydroxycholecalciferol, A Synthetic Analog of the Hormonal Form of Vitamin D_3

(1,25-dihydroxycholecalciferol/calcium absorption/bone calcium resorption/metabolic bone disease)

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ABSTRACT **1,25-Dihydroxycholecalciferol, the apparent active hormonal form of cholecalciferol (vitamin D_3), is formed from cholecalciferol by specific and sequential hydroxylations of the sterol at carbons 25 and 1. Recently, 1α-hydroxycholecalciferol was synthesized and we report on its biological activity in rachitic chicks. 1α-Hydroxycholecalciferol is identical in potency to 1,25-dihydroxycholecalciferol in stimulation of intestinal calcium absorption; either sterol elicits a near maximal effect at a dose of 0.3–0.6 nmol. The time-course of action of 1α-hydroxycholecalciferol also parallels that of the active metabolite 1,25-dihydroxycholecalciferol with a maximal increase in calcium transport occurring 5–10 hr after administration of sterol to vitamin D-deficient chicks. 6.5 nmol of 1α-hydroxycholecalciferol causes a doubling in calcium absorption in only 2–3 hr, which is the most rapid physiologic response yet detected for a vitamin D-sterol. 1α-Hydroxycholecalciferol is active also in enhancing bone calcium resorption and, like 1,25-dihydroxycholecalciferol, is at least 10 times as active as cholecalciferol in mobilizing bone calcium and raising plasma calcium concentration. It is concluded that 1α-hydroxycholecalciferol represents a synthetic analog of 1,25-dihydroxycholecalciferol that can be used both to study the mechanism of action of this hormone and as a therapeutic agent in the treatment of patients with certain metabolic bone diseases.**

Recent investigations indicate that cholecalciferol (CC) or vitamin D_3 is metabolized first to 25-hydroxycholecalciferol (25-OH-CC) in several tissues (1, 2) and this metabolite is further metabolized to 1,25-dihydroxycholecalciferol [1,25-$(OH)_2$-CC] in the kidney (3). 1,25-$(OH)_2$-CC is apparently the metabolite of cholecalciferol that promotes intestinal calcium absorption (4) and bone calcium resorption (5) and is considered to be the hormonal form of the vitamin. 1,25-$(OH)_2$-CC is the most active and fastest acting metabolite in both intestine (4) and bone (6, 7), and studies with radioactive cholecalciferol indicate that the metabolite functions by localizing in the nucleus of target cells (8, 9). Recent evidence suggests that 1,25-$(OH)_2$-CC binds initially to a specific cytosol receptor in the intestine (10) and is subsequently transported to the nuclear chromatin where it associates with a chromosomal receptor protein (11). It is probable that 1,25-$(OH)_2$-CC functions analogously to other sterol hormones (12–14) by inducing the synthesis of specific mRNA(s). The newly synthesized mRNA could code for specific proteins that are functional mediators of calcium translocation, such as the calcium-binding protein in intestinal mucosa (15).

25-OH-CC is an obligatory intermediate in the ultimate formation of 1,25-$(OH)_2$-CC, for the renal 1-hydroxylase enzyme will accept only 25-OH-CC as a substrate (Gagnon and Haussler, manuscript in preparation). In addition, 1-hydroxycholecalciferol is not found after injection of radioactive cholecalciferol into a vitamin D-deficient animal (16). These observations suggest that 1-hydroxycholecalciferol may not be a naturally occurring sterol. Barton, Hesse, Pechet, and Rizzardo (17) have recently completed the chemical synthesis of 1α-hydroxycholecalciferol (1α-OH-CC), and the present report describes the biological activity of 1α-OH-CC in rachitic chicks. This synthetic sterol is found to be equal in potency to 1,25-$(OH)_2$-CC with respect to stimulation of both intestinal calcium absorption and bone calcium resorption. Its pattern of action is virtually identical to that of 1,25-$(OH)_2$-CC. These data indicate that the *1-hydroxyl is the key functional group that confers enhanced vitamin D-activity.*

MATERIALS AND METHODS

Animals and Materials. Animals used in all experiments were White Leghorn cockerels that were raised for 3–4 weeks on a vitamin D-deficient diet (18). Cholecalciferol was purchased from Calbiochem. 1,25-(OH)-CC was produced enzymatically from crystalline 25-OH-CC (courtesy of Dr. John C. Babcock, Upjohn) by a modification (19) of the procedure of Lawson *et al.* (20). It was purified extensively by chromatography on columns of Sephadex LH-20 and Celite (19) until an ultraviolet spectrum of the preparation showed no contaminating materials. 1α-OH-CC was synthesized as described in detail elsewhere (17).

Structure Verifications and Quantitation of Sterols. Ultraviolet absorption studies were performed in distilled ethanol on a Cary model 15 spectrophotometer. Ultraviolet absorbance of all sterols was used as the method for accurate quantitation of doses for bioassay. A model RMU-GE Hitachi double-focusing mass spectrometer was used to analyze 5- to 10-μg portions of each sterol to confirm its chemical structure. Samples were directly introduced on the probe and continuous scanning was done from 80° to 150° above ambient.

Abbreviations: CC, cholecalciferol or vitamin D_3; 25-OH-CC, 25-hydroxycholecalciferol; 1α-OH-CC, 1α-hydroxycholecalciferol; 1,25-$(OH)_2$-CC, 1,25-dihydroxycholecalciferol.

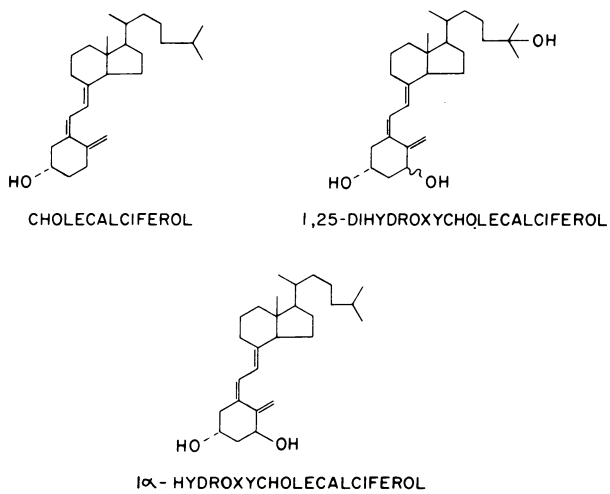

FIG. 1. Structures of cholecalciferol (vitamin D_3), 1,25-dihydroxycholecalciferol, and 1α-hydroxycholecalciferol. The designation of α- for the hydroxyl group on carbon 1 in 1α-hydroxycholecalciferol refers back to its stereochemical orientation in the corresponding provitamin form with a closed B-ring.

Bioassays. Calcium absorption stimulating activity of the various sterols was assayed by the method of Coates and Holdsworth (21). Plasma ^{45}Ca was assessed 45 min after oral administration of ^{45}Ca to vitamin D-deficient chicks that had received sterols (orally, dissolved in 1,2-propanediol) at prescribed intervals before the assay. 200 μl of plasma were added to 10 ml of Aquasol (New England Nuclear Corp.) and counted on a Beckman model LS-250 liquid scintillation spectrometer.

Bone calcium mobilizing activity of the sterols in question was carried out essentially as described by Hibberd and Norman (22). After chicks were raised for 3–4 weeks on the rachitogenic diet described earlier (18), the chicks were transferred for 4 days to an identical diet except that the calcium was omitted. Sterols were then administered orally in 1,2-propanediol and plasma calcium concentration was determined at appropriate intervals thereafter. An increase in plasma calcium level in chicks on this diet reflects a sterol-mediated increase in mobilization of bone. Plasma calcium was measured with the aid of a Beckman model 495 atomic absorption system. 0.2-ml Aliquots of plasma were mixed with 4.8 ml of aqueous diluent containing 10 g of $LaCl_3$, 20 ml of 11.6 N HCl, and 60 ml of *n*-butanol per liter. The mixture was clarified by centrifugation at 10,000 × *g* for 10 min, and the supernatant fluid was used for atomic absorption measurements.

RESULTS

Structure of 1α-OH-CC

The structures of cholecalciferol, 1,25-dihydroxycholecalciferol, and 1α-hydroxycholecalciferol are presented in Fig. 1. The synthetic 1α-OH-CC lacks a hydroxyl group at carbon 25, but is otherwise identical to the natural 1,25-$(OH)_2$-CC metabolite. Before bioassay the sterols were analyzed by ultraviolet absorption spectrophotometry to determine dosage and by mass spectrometry to verify chemical structure and stability. The ultraviolet absorption spectrum of synthetic 1α-OH-CC is shown in Fig. 2. Its spectrum is identical to that of cholecalciferol and 1,25-$(OH)_2$-CC, with a maximum at 265 nm and a characteristic trough at 228 nm (19). Mass spectrometric analysis of CC, 1α-OH-CC, and 1,25-$(OH)_2$-CC is depicted in Fig. 3. Respective parent molecular ions of *m/e* 384, 400, and 416 are seen for cholecalciferol and its mono- and dihydroxylated derivatives. Both crystalline 1α-OH-CC and biologically prepared 1,25-$(OH)_2$-CC yield fragments at *m/e* 287, 269 (287-H_2O), 251 (287-2H_2O), 152, and 134 (152-H_2O), indicating that the synthetic sterol, like the natural hormone, contains an additional oxygen atom in the A-ring. Thus, the spectra are essentially homologous and are completely consistent with the required structures (17, 19).

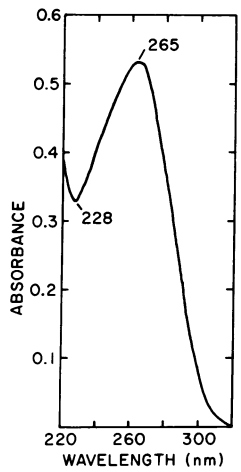

FIG. 2. Ultraviolet absorption spectrum of 1α-hydroxycholecalciferol.

Biological activity of 1α-OH-CC in stimulating intestinal calcium absorption

The most characteristic feature of the biological activity of 1,25-$(OH)_2$-CC is that it is capable of increasing calcium absorption with a maximum effect at 5–10 hr (4); its progenitor, cholecalciferol, requires 24–40 hr to elicit a maximal stimulation of calcium absorption. 1α-OH-CC was compared with 1,25-$(OH)_2$-CC in its ability to increase calcium absorption within 9 hr (Table 1). As little as 0.16 nmol of either sterol causes a significant increase in calcium absorption; maximal effects are

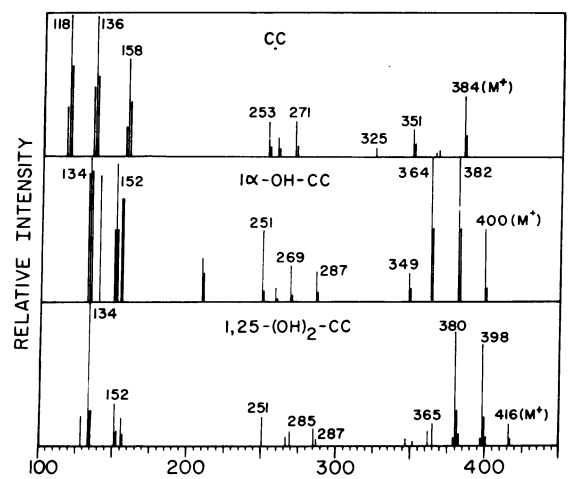

FIG. 3. Mass spectra of CC, synthetic 1α-OH-CC, and enzymatically generated 1,25-$(OH)_2$-CC.

TABLE 1. *Comparison of 1α-hydroxycholecalciferol and 1,25-dihydroxycholecalciferol in their ability to rapidly promote increased calcium absorption in chicks*

Sterol administered*	Amount of sterol (nmol)	Calcium absorption† (cpm ^{45}Ca per 0.2 ml of plasma)	Increment above control	% of maximal response
None (rachitic control)	0	315 ± 77	—	—
1α-OH-CC	0.16	533 ± 84	218	38
1,25-$(OH)_2$-CC		562 ± 58	247	43
1α-OH-CC	0.65	812 ± 156	497	86
1,25-$(OH)_2$-CC		834 ± 81	519	90
1α-OH-CC	6.5	895 ± 145	580	100

* Sterols were administered orally to rachitic chicks 9 hr before the assay of calcium absorption.

† Calcium absorption was assayed as described in *Methods*. Each number is the average of determinations on five separate animals (± SD). All groups treated with sterols are significantly different from rachitic control at $P < 0.01$.

seen at a dose of 0.65 nmol of the sterols. Of major significance is the equivalence in activity between 1α-OH-CC and 1,25-$(OH)_2$-CC. On a mole basis, 1α-OH-CC and 1,25-$(OH)_2$-CC produce virtually identical biologic responses. Raising the dose of 1α-OH-CC to a level of 6.5 nmol does not significantly increase the response; thus, a maximum of 0.65 nmol of 1α-OH-CC is apparently sufficient to saturate the hormone receptors in the intestine.

Time-course of action of 1α-OH-CC

Fig. 4 shows the time-course of action of 0.5 nmol of CC, 1,25-$(OH)_2$-CC, or 1α-OH-CC in stimulating calcium absorption in rachitic chicks. 1α-OH-CC induces a rapid increase in calcium absorption with a maximal effect occurring in 5–10 hr. The temporal response to the 1α-OH-CC is coincident with that of 1,25-$(OH)_2$-CC; both sterols induce changes in calcium transport far faster than does cholecalciferol. It may also be of

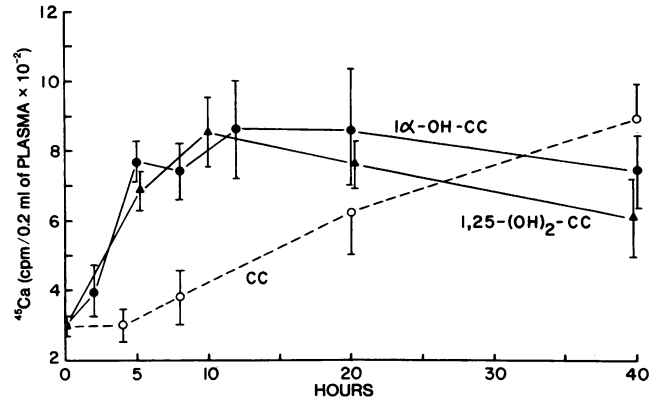

FIG. 4. Time-course of induction of intestinal calcium absorption in rachitic chicks after an oral dose of 0.5 nmol of CC (O- - -O), 1α-OH-CC (●—●), or 1,25-$(OH)_2$-CC (▲—▲). Calcium absorption was assayed as described in *Methods*. Each number represents the average of five separate animals ± SD.

significance that the 1α-OH-CC sterol appears to have a slightly more sustained action than the natural hormone, 1,25-$(OH)_2$-CC (Fig. 4).

The results illustrated in Fig. 4 indicate that there is about a 2-hr lag in response to 1α-OH-CC or 1,25-$(OH)_2$-CC. However, the availability of large quantities of the synthetic 1α-OH-CC allows us to further probe this latent period. Fig. 5 demonstrates the time-course of action of a relatively large dose of 6.5 nmol administered orally. Again, 1α-OH-CC acts much more rapidly than cholecalciferol. There is a doubling of calcium absorption in only 2–3 hr with this dose of 1α-OH-CC. Although this represents the most rapid and dramatic increase in calcium absorption thus far reported for a vitamin D-sterol, there exists a distinct lag of 60–90 min in the response to 6.5 nmol of 1α-OH-CC, which may be consistent with the functioning of this sterol through the control of gene expression and protein synthesis.

Bone calcium mobilizing activity of 1α-OH-CC

The bone calcium resorbing activity of 1α-OH-CC was assessed by administering the sterol to rachitic chicks that had been placed on a low calcium diet for 4 days. Increases in

TABLE 2. *Bone calcium mobilizing activity of 1α-hydroxycholecalciferol in rachitic chicks*

Sterol administered*	Amount of sterol (nmol)	Time† (hr)	Plasma calcium concentration‡ (mg/100 ml)	Increment above control	% of maximal response
None(rachitic-low calcium control)	0	—	5.3 ± 0.7	—	—
Cholecalciferol(CC)	6.5	8	6.4 ± 0.6	1.1	25
1,25$(OH)_2$-CC	0.65	8	7.1 ± 0.8	1.8	41
1α-OH-CC	0.65	8	6.9 ± 0.4	1.6	36
1α-OH-CC	6.5	2	6.8 ± 0.4	1.5	34
1α-OH-CC	6.5	5	6.5 ± 0.6	1.2	27
1α-OH-CC	6.5	8	8.7 ± 1.4	3.4	77
1α-OH-CC	6.5	16	9.7 ± 0.7	4.4	100

* Sterols were administered orally to rachitic chicks that had been on a low calcium diet for 4 days (see *Methods*).

† Time between oral dose of sterol and assay of plasma-calcium concentration.

‡ Each number is the average of five animals ± SD. All values are significantly different from control at $P < 0.05$.

plasma calcium concentration of these chicks is largely a measure of the bone calcium mobilizing activity of the sterol (22). Both 1,25-$(OH)_2$-CC and 1α-OH-CC are considerably more active than cholecalciferol 8 hr after administration (Table 2). Since 0.65 nmol of natural hormone or 1α-OH-CC elicits a greater increase in plasma calcium than 6.5 nmol of cholecalciferol (Table 2), the hydroxylated derivatives are estimated to be at least 10-times as active as the parent cholecalciferol. 1α-OH-CC appears to be equal to 1,25-$(OH)_2$-CC in bone calcium mobilizing activity (Table 2) as well as in stimulating calcium transport from the intestine (Table 1). With a larger dose of 6.5 nmol of 1α-OH-CC, significant increases in plasma calcium are seen as early as 2 hr, and by 16 hr the plasma calcium level is strikingly increased to a near normal level of 9.7 mg/100 ml (Table 2).

DISCUSSION

The data show that in chicks, 1α-hydroxycholecalciferol is equal in efficacy to 1,25-$(OH)_2$-CC, the active metabolite of vitamin D_3. 1α-OH-CC also possesses considerable activity when assayed in rats (M. Pechet, unpublished). 1α-OH-CC exerts an effect on both of the established vitamin D-target organs—the intestine and bone—and its rapid activity in stimulating intestinal calcium absorption and bone mineral mobilization suggest that it may be a useful analog of the vitamin.

The biological activity of synthetic 1α-OH-CC provides evidence that the stereochemical orientation of the hydroxyl group on carbon 1 is α- in the natural metabolite 1,25-$(OH)_2$-CC. This concept was originally derived from the studies of Lawson *et al.* (23) who found that 1α-^{3}H was stereospecifically lost from cholecalciferol during metabolism to 1,25-$(OH)_2$-CC. Semmler *et al.* (24) have recently synthesized 1α,25-dihydroxycholecalciferol and thus independently have demonstrated the 1α-orientation for the natural metabolite 1,25-$(OH)_2$-CC.

The possibility arises that 1α-OH-CC may exert its hormonal effect largely through conversion into 1,25-$(OH)_2$-CC. However, such an obligatory transformation is rather difficult to reconcile with the observations that 1α-OH-CC is essentially equipotent to 1α,25-$(OH)_2$-CC (Table 1) and that the effects of both substances follow the same time-course (Fig. 4), demanding, as it would, an extremely rapid and efficient transformation of exogenous 1α-OH-CC into 1α,25-$(OH)_2$-CC. On this basis, the biological effects we have observed may arise from 1α-OH-CC acting *per se*. Further, this assumption leads to the inference that 1α-hydroxyl group is the structural feature required for full expression of hormonal activity. If this is true, it is then appropriate to question the function of the 25-hydroxylation of cholecalciferol. One possibility is that 25-hydroxylation of cholecalciferol creates a sterol that can be further metabolized to a hormonal form in a controlled fashion in a specific organ, the kidney. The 1-hydroxylase enzyme has been reported to catalyze the synthesis of 1,25-$(OH)_2$-CC according to the calcium needs of the animal (25) and this regulation may be mediated by parathyroid hormone (26). As the presence of the 25-hydroxyl group appears to be necessary for binding the sterol to the highly specific 1-hydroxylase enzyme in the kidney, the 25-hydroxyl may be crucial to control the formation of hormone, while the 1α-hydroxyl may be the key element in binding to the target-tissue receptors.

On the other hand, evidence exists to support the notion that the 1α-OH-CC sterol may be 25-hydroxylated to 1,25-$(OH)_2$-CC. Haussler *et al.* (4) showed that 25-OH-CC acts only slightly faster than cholecalciferol while 1,25-$(OH)_2$-CC functions far more rapidly than either precursor sterol. This finding suggests that the rate-limiting step in vitamin D metabolism *in vivo* is 1-hydroxylation and that 25-hydroxylation proceeds at a relatively rapid rate. Also, cholecalciferol-25-hydroxylase has been found in several tissues, including the intestine (2). It is not known whether 1α-OH-CC can serve as a substrate for the 25-hydroxylase enzyme, but a similar analog, dihydrotachysterol$_3$, is converted to 25-hydroxy-dihydrotachysterol$_3$ in experimental animals (27). Direct studies with radioactive 1α-OH-CC to assess its metabolism and capability of binding to specific 1,25-$(OH)_2$-CC receptors (10) are required to determine if 1α-OH-CC is an efficient precursor of 1,25-$(OH)_2$-CC or is functioning, *per se*, as an ideal analog of the natural hormone.

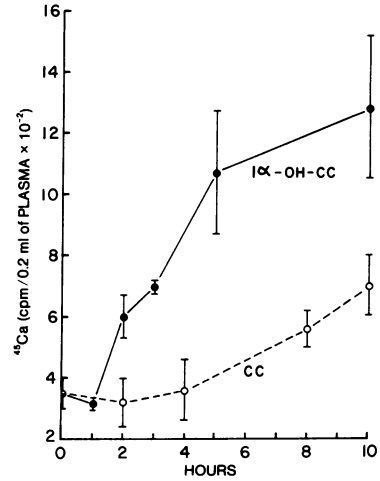

FIG. 5. Early time-course of action of 6.5 nmol of either 1α-OH-CC (●—●) or CC (○- - -○) in promoting intestinal calcium transport. Sterols were administered orally and calcium absorption was measured as described in *Methods*. Each number is the average of separate determinations on five animals ± SD.

Our study suggests that 1α-OH-CC should prove to be an important compound in studying the mechanism of action of cholecalciferol derivatives. The synthetic sterol may be especially useful in elucidating the nature of specific receptors for vitamin D-active sterols. In addition, its rapid activity makes it an ideal probe in the search for the initial biochemical events in the mode of action of vitamin D. Since the 1α-OH-CC can be readily synthesized in quantity it may become an effective agent in treating certain disorders of calcium metabolism and dysfunction in vitamin D metabolism, such as renal osteodystrophy (28), hypoparathyroidism (29), and vitamin D-resistant rickets.

NOTE ADDED IN PROOF

Recently, Holick *et al.* [(1973) *Science* **180**, 190–191], have reported that 1α-OH-CC is comparable in potency to 1,25-$(OH)_2$-CC in vitamin D-deficient rats. These investigators also raised the question of whether 1α-OH-CC was converted *in vivo* to the natural 1,25-$(OH)_2$-CC hormone. Based upon recent work in our laboratory using the receptor for 1α,25-$(OH)_2$-CC, we have determined that 1α-OH-CC probably functions by metabolic conversion to 1α,25-$(OH)_2$-CC.

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