Biological Activity of 1,25-Dihydroxycholecalciferol*

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ABSTRACT: A biologically active vitamin D_3 metabolite (1,25-dihydroxycholecalciferol) was generated in deficient chicks and isolated using Sephadex LH-20 chromatography. The use of silicic acid chromatography for the isolation of 1,25-dihydroxycholecalciferol from chicks dosed with high levels of vitamin D (6.5 μmoles) resulted in a heterogeneous metabolite fraction, as revealed by LH-20 chromatography. The concentration of 1,25-dihydroxycholecalciferol in the intestine was found to be constant at 13 pmoles/chick, regardless of vitamin dosage. Use of a sensitive *in situ* technique revealed that 1,25-dihydroxycholecalciferol (325 pmoles) stimulated intestinal calcium transport more rapidly and to a greater extent than an equivalent dose of 25-hydroxycholecalciferol. In contrast, 1,25-dihydroxycholecalciferol appeared less than half as effective as 25-hydroxycholecalciferol when assayed for antirachitic activity in the rat. This result was not unexpected since 1,25-dihydroxycholecalciferol has an apparently short half-life *in vivo*. Serum calcium concentration of rats on a low-calcium, vitamin D deficient diet was also increased by 1,25-dihydroxycholecalciferol (65 pmoles), but to a lesser degree than a similar dose of 25-hydroxycholecalciferol. Results indicate that the 1,25-dihydroxycholecalciferol is the apparent metabolically active form of the vitamin in catalyzing intestinal calcium transport.

It had been assumed for some 30 years following the elegant work concerning the structural identification of vitamin D_2 (Askew *et al.*, 1931; Windaus *et al.*, 1936) and D_3 (Schenck, 1937) that vitamin D *per se* was the functional form of the vitamin. This question remained unexamined until the synthesis of moderate to high specific activity radioactive vitamin D_3 (Norman and DeLuca, 1963; Neville and DeLuca, 1966). It was first possible to demonstrate with the moderate specific activity material that indeed biologically active metabolites of vitamin D are formed *in vivo* (Norman *et al.*, 1964). However, the concept of a metabolically active form of vitamin D did not make its appearance until Lund and DeLuca (1966) demonstrated quite conclusively the existence of a metabolite fraction that possessed biological activity equivalent to that of the parent vitamin. Of great importance was the fact that this fraction contained a metabolite or metabolites which acted more rapidly than the parent vitamin (Morii *et al.*, 1967). Blunt *et al.* (1968a) subsequently isolated in pure form the major metabolite of vitamin D from the plasma of pigs and identified its structure as 25-HCC.[1] Not only did this metabolite exceed the biological activity of vitamin D_3, but it acted more rapidly in inducing intestinal calcium transport and bone mineral mobilization (Blunt *et al.*, 1968b). Also important was the finding that 25-HCC acts directly in isolated target tissues of vitamin D whereas vitamin D_3 itself does not (Trummel *et al.*, 1969; Olson and DeLuca, 1969), demonstrating that vitamin D must be hydroxylated in the 25 position before it is effective. The fact that 25-HCC is the major metabolite of vitamin D in the blood (Ponchon and DeLuca, 1969a) provided evidence that this could well represent a hormonal form of the vitamin. The 25-HCC is synthesized by a very tightly controlled enzymatic process in which the liver serves as a type of endocrine organ for the secretion of active vitamin D (Ponchon and DeLuca, 1969b; Ponchon *et al.*, 1969; Horsting and DeLuca, 1969). The synthesis of radioactive 25-HCC (Suda *et al.*, 1971) next allowed the study of whether 25-HCC itself is the active form or whether it must be converted further before it is effective. Accordingly, Cousins *et al.* (1970a,b) demonstrated that in fact there is a very rapid appearance of a polar metabolite in intestine and in other tissues which could well represent the metabolically active form. This metabolite was labeled as peak V because of its behavior on silicic acid columns. The generation of this metabolite from vitamin D_3 was shown by Haussler *et al.* (1968) followed by Lawson *et al.* (1969) and Ponchon and DeLuca (1969a). Myrtle *et al.* (1970) have reported a marked effect of this metabolite on intestinal calcium absorption. More recently Holick *et al.* (1971a,b) and Lawson *et al.* (1971) have identified this polar metabolite as 1,25-DHCC.

Examination of the biological properties of 1,25-DHCC seemed of great importance since it may represent the metabolically active form in the intestine. At present there is uncertainty concerning the biological activity of 1,25-DHCC, due in part to the questions which arose regarding the methods used in estimating the metabolites' effect on intestinal calcium transport. Therefore, a reliable *in situ* method for examining intestinal calcium transport response in the chicken to 1,25-DHCC and other forms of vitamin D was developed. Using this it was possible to show that 1,25-DHCC initiates intestinal calcium absorption much more rapidly than does either 25-HCC or vitamin D_3. However, 1,25-DHCC is less than half as active as 25-HCC in the cure of rickets in rats and is somewhat less effective than 25-HCC in stimulating bone mineral mobilization. These results suggest that 1,25-DHCC may well represent the metabolically active form of the vitamin, at least in the intestine. It is the purpose of this report to present these results.

Materials and Methods

Chicks. White Leghorn cockeral chicks were obtained from

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[1] Abbreviations used are: 25-HCC, 25-hydroxycholecalciferol; 1,25-DHCC, 1,25-dihydroxycholecalciferol; 21,25-DHCC, 21,25-dihydroxycholecalciferol.

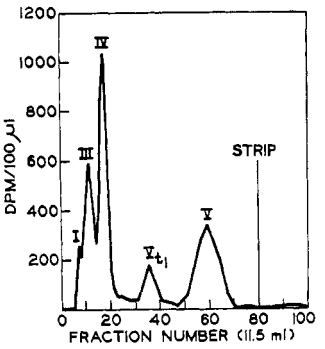

FIGURE 1: Sephadex LH-20 column (65% chloroform in Skellysolve B) chromatographic profile of intestinal extract from chicks dosed (iv) with 6.25 µg (250 IU) of [1,2-^{3}H]vitamin D_3 24 hr earlier.

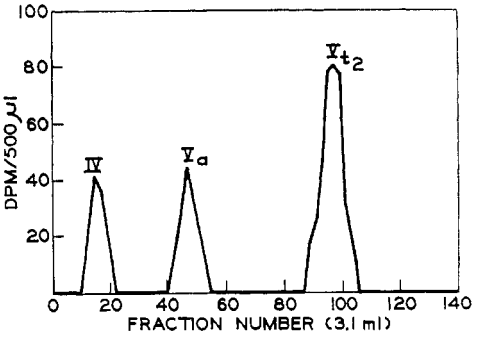

FIGURE 3: Chromatography of silicic acid peak V region on a Sephadex LH-20 (65% chloroform in Skellysolve B) column. The peak V region was obtained from chicks given 2.5 mg (100,000 IU) of [^{3}H]vitamin D_3 each orally 24 hr earlier.

Northern Hatcheries of Beaver Dam, Wis. They were maintained in cages at 38°, shielded from ultraviolet light, and fed *ad libitum* one of two rachitogenic diets. Diet A was a corn–soy protein diet which produced rachitic symptoms at approximately 14 days of age (Thornton and Omdahl, 1969). Diet B was a vitamin D deficient purified diet composed of the following in per cent: soy protein, 25.6; sucrose, 56; DL-methionine, 0.6; glycine, 0.4; cellulose, 3; cottonseed oil, 4; $CaHPO_4$, 0.52; KH_2PO_4, 1.55; $CaCO_3$, 2.28; NaCl, 0.8, with the trace salts and vitamins added as previously reported (Imrie *et al.*, 1967). Chickens used for generation of 1,25-DHCC were fed diet A for three weeks followed by diet B for one week. Bioassay chicks received diet B throughout the course of the experiment (3.5 weeks).

Rats. Male, weanling rats were purchased from the Holtzman Co., Madison, Wis. They were fed *ad libitum* either the rachitogenic diet of Steenbock and Black (1924) with added crystalline water-soluble vitamins as described by Suda *et al.* (1970c) or the low-calcium, vitamin D deficient diet of Blunt *et al.* (1968b). They were housed individually in hanging wire cages. The rachitic rats were used for the antirachitic line-test assay (U. S. Pharmacopoeia, 1955). The rats fed the low-calcium diet were used for the bone mineral mobilization test (Blunt *et al.*, 1968b) of vitamin D activity.

Radioactive Vitamin D_3. [1,2-^{3}H]Vitamin D_3 (0.6 Ci/mmole) was prepared as previously described (Neville and DeLuca, 1966) while [4-^{14}C]vitamin D_3 (19.2 mCi/mmole) was a gift from the Philips-Duphar Company of the Netherlands. Both preparations were purified using Sephadex LH-20 chromatography (Holick and DeLuca, 1971) and dissolved either in cottonseed oil for oral administration or in 95% ethanol for iv injection (0.05 ml).

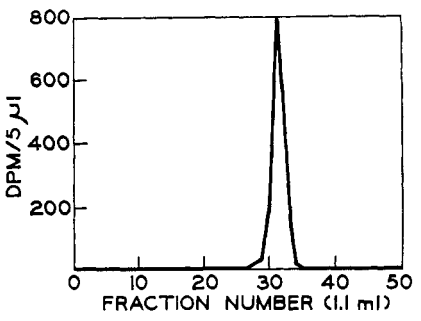

FIGURE 2: Rechromatography of peak V (*i.e.*, 1,25-DHCC) from Figure 1 on a Sephadex LH-20 column in methanol.

Preparation of Extracts Containing Vitamin D Metabolites. A. CHICKS GIVEN 6.25 µg OF (250 IU) VITAMIN D_3. Four-week-old, vitamin D deficient chickens were dosed iv with 6.25 µg of [1,2-^{3}H]vitamin D_3 (240,000 dpm/µg) and killed 24 hr later. The whole small intestine was excised, flushed with ice-cold distilled water, and rapidly frozen on solid CO_2. The combined tissue from 600 chickens was extracted with chloroform and methanol as previously described (Lund and DeLuca, 1966).

B. CHICKS GIVEN 2.5 mg (100,000 IU) OF VITAMIN D_3. The procedure was identical with the 6.25-µg/chick experiment except as noted. Vitamin D deficient chickens (100) were injected iv with 2.5 mg of [1,2-^{3}H]vitamin D_3 (1460 dpm/µg) and killed 24 hr later.

Chromatography of the Intestinal Extracts. The intestinal lipid extract from chicks dosed with 6.25 µg (250 IU) of [^{3}H]vitamin D_3 was dissolved in a minimal volume of 65% chloroform in Skellysolve B (a petroleum fraction, bp 67–69°) and applied to a 60-g Sephadex LH-20 column (2.0 × 60 cm) which contained 2 cm of Celite on top of the gel bed. The column was equilibrated and then eluted with 800 ml of the solvent mixture, resulting in the detection of five radioactive peaks (Figure 1). The column was stripped with 200 ml of 75% chloroform in Skellysolve B. Fractions of 11.5 ml were collected and a 0.1-ml aliquot of each was taken to determine radioactivity. Peaks V and V_{t_1} (Figure 1) were then rechromatographed individually on a 20-g (1.1 × 60 cm) Sephadex LH-20 column (65% chloroform in Skellysolve B) followed by a MeOH Sephadex LH-20 column as previously described (Suda *et al.*, 1970a,b) (Figure 2). Cochromatography of peaks V and V_{t_2} (Figure 4) was carried out using the 20-g LH-20 column equilibrated with 65% chloroform in Skellysolve B.

The intestinal extract from chicks dosed with 2.5 mg (100,000 IU) of [^{3}H]vitamin D_3 was initially applied to a large multibore silicic acid column as previously described (Suda *et al.*, 1970a,b). The peak V region from this column, which appeared homogeneous on silicic acid was applied to a 20-g Sephadex LH-20 column described above (Holick and DeLuca, 1971). The column was eluted with 300 ml of the solvent (65% chloroform in Skellysolve B). Fractions of 3.1 ml were collected and an aliquot of each was taken for ^{3}H determination. Three radioactive peaks were detected, showing the silicic acid peak V region to be heterogeneous (Figure 3).

Double-Label [$^3H/^{14}C$] Experiment. Three vitamin D deficient chicks were injected iv (6.25 µg/chick) with a mixture of [1,2-^{3}H]- (2.5×10^6 dpm/µg) and [4-^{14}C]- (1×10^5 dpm/µg) vitamin D_3 which had a $^3H/^{14}C$ ratio of approximately 5.3:1. Chicks were killed at 24 hr and the peak V isolated using a

65% chloroform in Skellysolve B LH-20 column (20 g, 1.1 × 60 cm) as described above. Radioactivity was estimated and the $^{3}H/^{14}C$ ratio determined by use of a Packard Model 3375 liquid scintillation spectrometer.

Bioassay of Vitamin D Metabolites. The enhancement of intestinal calcium absorption in vitamin D repleted chickens was the basis of the bioassay. Calcium (*i.e.*, ^{45}Ca) absorption was estimated by using a duodenal loop *in situ* method. Vitamin D deficient chicks weighing between 170 and 190 g were dosed (oral or iv) with 25-HCC, vitamin D_3, or peak V metabolite at various time periods prior to experimentation. Chicks were fasted 15 hr before experimentation and then anesthetized with chloral hydrate (35 mg/100 g body weight, im). A 10-cm portion of the duodenal loop was ligated and its lumen rinsed with 5 ml of a phosphate-free bicarbonate solution (120 mM NaCl, 4.9 mM KCl, and 9.2 mM $NaHCO_3$; pH adjusted to 7 with CO_2) after which the proximal ligature was tied off. The distal ligature was tightened around a blunted 18 gauge needle through which 0.2 ml of phosphate-free [^{45}Ca]calcium bicarbonate solution (0.5 μCi of ^{45}Ca/ml; 10 mM $CaCl_2$) was injected into the lumen of the duodenal loop. Following withdrawal of the needle the ligature was secured and the loop replaced in the peritoneal cavity for 30 min at which time the animal was killed. The loop was rapidly excised and subsequently dry-ashed in porcelain crucibles at 600° for 48 hr. The ashed product was dissolved in 2 N HCl, neutralized with 2 N Tris and 0.05-ml aliquots spotted on filter paper disks (2.3-cm diameter). Radioactivity was estimated by placing the paper disk in a 20-ml scintillation counting vial, adding 10 ml of toluene counting solution (Neville and DeLuca, 1966), and counting in a Tri-Carb liquid scintillation spectrometer (Model 3375). Results were expressed as per cent ^{45}Ca absorption and were calculated using the following formula: $\% \ ^{45}Ca_{Abs} = [1 - {}^{45}Ca_R/{}^{45}Ca_A](100)$, where $^{45}Ca_R$ is the amount of ^{45}Ca remaining in the duodenal loop following the *in situ* incubation and $^{45}Ca_A$ is the amount of ^{45}Ca initially added to the loop preparation.

Antirachitic Activity Assay. Rachitic rats were injected iv with either 260 pmoles (4 IU) of standard vitamin D_3, 260 pmoles of 25-HCC, or 260 pmoles of 1,25-DHCC iv in 0.02 ml of 95% ethanol. Controls received only 0.02 ml of 95% ethanol. Seven days later the rats were killed, their radii and ulnae were removed and stained with 1.5% silver nitrate solution, and the apparent calcification was scored. The antirachitic activity was estimated according to the U. S. Pharmacopoeia (1955).

Bone Mineral Mobilization Activity Assay. The low-calcium, vitamin D deficient rats were injected (iv) with 0.02 ml of ethanol containing 65 pmoles of either 25-HCC or peak V. Controls received 0.02 ml of ethanol carrier. Blood samples were taken at 6, 24, 48, 72, and 96 hr for calcium determination. In another experiment identically carried out, a blood sample was taken 12 hr after either 1,25-DHCC or 25-HCC. No response was observed at 12 hr to either peak V or 25-HCC.

Serum Calcium Determination. Calcium was estimated using atomic absorption spectrophotometry (Perkin-Elmer Model 214). Serum samples (0.05 ml) were dissolved in 2 ml of 0.1% $LaCl_3$ and assayed directly for calcium. Blank and standard solutions were made to approximate diluted serum by the addition of NaCl (3.42 mM) and KCl (0.19 mM).

Results

In order to estimate the amount of 1,25-DHCC it was necessary to use known specific activity radioactive vitamin D_3 in vitamin D deficient chicks for generation of 1,25-DHCC *in vivo*. This assumes that the specific radioactivity of the metabolite is the same as the parent vitamin. The vitamin D_3 in the present experiments was labeled in the 1 and 2 positions which necessitated a preliminary experiment concerning possible ^{3}H loss in the 1 position (Lawson *et al.*, 1969) during generation of 1,25-DHCC. Injection (iv) of vitamin D deficient chicks with a mixture of [1,2-^{3}H]- and [4-^{14}C]cholecalciferol ($^{3}H/^{14}C$ of 5.3:1) resulted in a decreased $^{3}H/^{14}C$ ratio from 5.3 to 2.6 for the peak V metabolite (Table I), in agreement with Lawson *et al.* (1969). This was taken into account by decreasing the specific activity by a factor of two when calculating the amount of generated peak V metabolite.

TABLE I: Loss of Tritium During the Conversion of [1,2-^{3}H]-Vitamin D_3 to 1,25-DHCC in Intestine.

Compound[a]	$^{3}H/^{14}C$
Vitamin D_3	5.4
25-HCC	5.2
1,25-DHCC	2.6
Peak V_{t_1}	5.2

[a] A mixture of vitamin D_3 labeled either at positions 1 and 2 with ^{3}H or position 4 with ^{14}C was given iv to rachitic chicks and 24 hr later the mucosa from the small intestine was removed, extracted, and chromatographed on LH-20.

Generation of peak V metabolite in sufficient quantity to perform biological measurements required large groups of deficient chicks (*i.e.*, 600 chicks/group) due to a metabolite yield of 13 pmoles per chick (or approximately 1 ng/g of tissue).

Sephadex LH-20 column chromatography proved to be a valuable asset affording excellent separation of vitamin D_3 metabolites.[2] Peaks V and V_{t_1} could be isolated free of less polar metabolites (Figure 1) following chromatography on the LH-20 column (60 g, 65% chloroform in Skellysolve B). Both metabolites were individually rechromatographed on a smaller LH-20 column (20 g) (65% chloroform in Skellysolve B) and then on a methanol LH-20 column (20 g) (Suda *et al.*, 1970a,b). Each compound migrated as a single peak on the methanol column, examplified by the profile in Figure 2.

Preliminary studies in our laboratory employing silicic acid chromatography and various vitamins D_3 levels, revealed that high dosages (*e.g.*, 100,000 IU or 6.5 μmoles) could effect an increase in production of peak V metabolite. However, the peak V from silicic acid was heterogeneous and could be resolved into three radioactive peaks by Sephadex LH-20 column chromatography (Figure 3). One of the peaks (V_{t_2}) migrated in the peak V region; however, cochromatography indicated it was different from peak V (Figure 4). The peak V metabolite was not detected in chicks dosed with the large amount of vitamin D_3, due to the low specific activity of the preparation; strengthening the concept that 1,25-DHCC is present in the intestine only to a finite amount (about 1 ng/g) regardless of vitamin D dosage.

The *in situ* method for detection of changes in calcium

[2] In our laboratory the identified metabolites of vitamin D_3 and their corresponding peak numbers are as follows: vitamin D_3 ester, peak I; vitamin D_3 or cholecalciferol, peak III; 25-HCC, peak IV; 21,25-DHCC, peak Va; 1,25-DHCC, peak V.

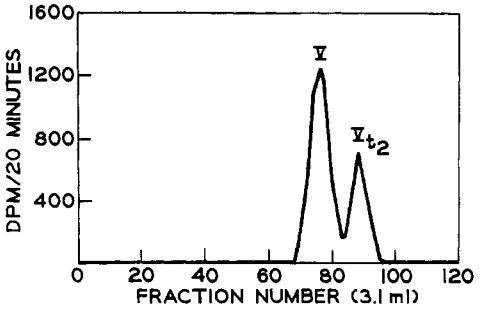

FIGURE 4: Cochromatography of peaks V from Figure 1 and V_{t_2} from Figure 3 using Sephadex LH-20 (65% chloroform in Skellysolve B).

absorption proved to be quite sensitive (Figure 5). As little as 195 pmoles of 25-HCC gave a significant increase in calcium absorption. The retrograde movement of calcium (*i.e.*, blood to intestine) ranged between 0.7 and 1.4% of the calcium placed in the intestine, a factor which added negligible error to the calculations. A time-course profile was obtained for vitamin D_3, 25-HCC, and 1,25-DHCC (Figure 6). It was observed that the effect of 25-HCC preceeded that of vitamin D_3 by approximately 5 hr, both compounds demonstrating similar calcium absorption profiles. Such results complement earlier studies using rats (DeLuca, 1969). The 1,25-DHCC, however, demonstrated strikingly novel characteristics by superseding 25-HCC in brevity of lag time and extent of response of calcium absorption. The 1,25-DHCC gave a maximal response at approximately 10 hr, which was twice the maximal response of 25-HCC as measured at 24 hr and then declined until equal in activity to 25-HCC at 24 hr (Table II). Two other metabolites (*i.e.*, V_{t_1} and V_{t_2}) isolated by Sephadex LH-20 chromatography failed to stimulate intestinal calcium absorption (Table II).

Since 1,25-DHCC gave a faster and larger response than either 25-HCC or vitamin D_3, it became of interest to see if a similar situation existed for the polar metabolite concerning antirachitic and bone mobilization activity.

Using the rat line test, the 1,25-DHCC assayed only 0.4 as effective as 25-HCC in curing rickets (Table III). Peak V_{t_2}, isolated from high vitamin D dosed chicks, demonstrated no detectable activity in the cure of rickets.

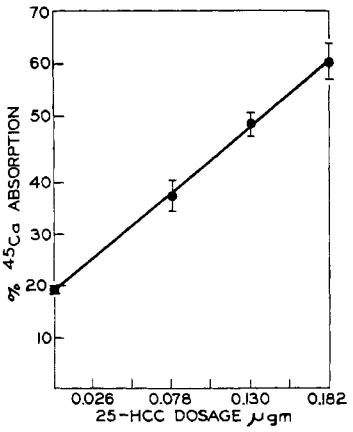

FIGURE 5: Dose-response curve for 25-HCC and calcium absorption in chicks. Chicks were injected iv 24 hr prior to use. Each point represents the average of 6 chicks. The vertical bars represent standard error.

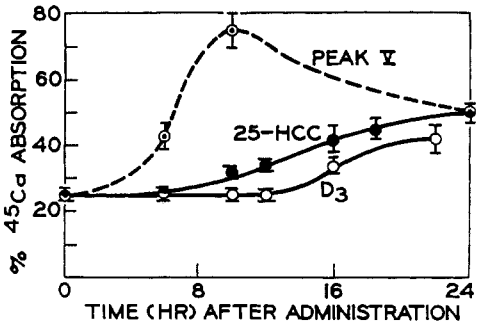

FIGURE 6: Response of chick intestinal calcium absorption to 25-HCC, vitamin D_3, and peak V (1,25-DHCC). Each point represents the average of 4–7 chicks ± standard error. (●———●) 325 pmoles given iv; (○———○) 325 pmoles given iv; (⊙———⊙) 325 pmoles administered orally.

Bone mobilization activity was conducted using small doses of 25-HCC and 1,25-DHCC (*i.e.*, 65 pmoles or ~1 IU). Serial blood samples revealed a significant rise in serum calcium at 24 hr and 72 hr for both the 25-HCC and 1,25-DHCC, however, the 25-HCC response was significantly larger ($p < 0.02$) at both time periods (Figure 7). The serum calcium showed a maximal increase of approximately 3 mg/100 ml of serum for both 25-HCC and 1,25-DHCC when measured at 48 hr, whereas at 96 hr both groups had returned to control levels. These results demonstrate that 1,25-DHCC does possess bone mobilizing activity in contrast to another report (Kodicek *et al.*, 1970).

Discussion

A highly refined multibore silicic acid chromatographic system and a new liquid–liquid Celite partition chromatography system (Suda *et al.*, 1970a) have made it clear that the peak V metabolite fraction from hog and chick plasma is extremely heterogeneous. Two of these metabolites have been recently isolated in pure form and identified as 21,25- and 25,-26-dihydroxy derivatives of the parent vitamin (Suda *et al.*,

TABLE II: Stimulation of Intestinal Calcium Absorption by 25-HCC, 1,25-DHCC, and Peak V Metabolites.

Compound	Dosage (pmoles)	Dosage Method	*ta* (hr)[a]	RTAM (*ta*)/25-HCC (t_{24})[b,c]
25-HCC	195–455	Iv and oral	24	1.00
Peak V_{t_1}	227	Oral	24	0
Peak V_{t_2}	258	Iv	24	0
1,25-DHCC	455	Iv	24	0.72
1,25-DHCC	455	Oral	24	1.09
1,25-DHCC	325	Oral	24	0.97
1,25-DHCC	325	Oral	10	2.00

[a] *ta* represents: time of assay following dosage. [b] (Net % ^{45}Ca absorption for metabolite at time *ta*)/(net % ^{45}Ca absorption for 25-HCC at time 24 hr) where net % ^{45}Ca absorption = % ^{45}Ca absorption for metabolite − % ^{45}Ca absorption for control. [c] RTAM = relative transport activity metabolite.

TABLE III: Antirachitic Activity of 1,25-DHCC in Rats.

Compound	Activity (IU/nm)
Vitamin D_3	15.4 ± 0.1[a]
25-HCC	21.5 ± 0.1
1,25-DHCC	8.1 ± 0.2
Peak V_{t_2}[b]	0

[a] Standard error. Each value represents the average of 7 animals. [b] See Figure 4.

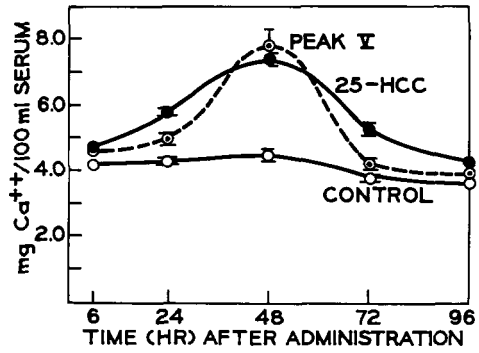

FIGURE 7: Plasma calcium response of vitamin D deficient rats on a low-calcium diet to a 65-pmole dose of 25-HCC or peak V (1,25-DHCC). The vertical bars represent the standard error of the mean from 4 to 6 animals.

1970a,b). Recently a new chromatographic procedure using Sephadex LH-20 in a mixed solvent of chloroform in Skellysolve B also demonstrated the heterogeneity of rat plasma peak V (Holick and DeLuca, 1971). More importantly, it showed that the peak V in the intestine which was generated from 0.25 μg of vitamin D_3 in chicks was homogeneous (*i.e.*, 1,25-DHCC), whereas at a dose of 2.5 mg of vitamin D_3 the peak V region, which appeared to be homogeneous on silicic acid, was resolved into three distinct peaks (Figure 3). One of the peaks (Va) was shown to be 21,25-DHCC by cochromatography in two different chromatographic systems (M. F. Holick, T. Suda, and H. F. DeLuca, 1970, unpublished data).

Lawson *et al.* (1969) initially observed that the peak V[3] metabolite which loses the 1-^{3}H cannot be increased in the intestine by increasing the dose of vitamin D_3. This observation has also been confirmed by Norman and Midgett (1970) and by our laboratory (this report). The discrepancies involving the biological activity of peak V appear to be due in part to the heterogeneity of the metabolite fraction. In particular, at high doses of vitamin D_3 three other metabolites predominate (peaks V_{t_1}, V_{t_2}, V_a). Since silicic acid cannot separate these three metabolites from the desired peak V, the apparent recovery of peak V from silicic acid is much higher than predicted. Ponchon *et al.* (1970) reported that the peak V metabolite isolated from the intestine is only about 50% as active as vitamin D_3 in intestinal Ca^{2+} absorption. Because they dosed the chicks with 250 μg, the recovered "apparent" peak V from the silicic acid column was about 10 times higher than would be expected based on 1 ng/g of tissue. Since V_{t_1} and V_{t_2} have no biological activity for intestinal Ca^{2+} transport, these metabolites diluted the effect of the peak V. Also 21,25-DHCC elicits an intestinal Ca^{2+} transport response that is one-half of that of vitamin D_3. Only when physiological doses of vitamin D_3 are given to the animals is a homogeneous peak V (*i.e.*, 1,25-DHCC) obtained on silicic acid chromatography of intestinal extracts.

Previous studies concerning 1,25-DHCC and stimulation of calcium absorption have used a method which estimates disappearance of ^{45}Ca from the intestine by measuring its appearance in blood (Ponchon *et al.*, 1970; Myrtle *et al.*, 1970; Lawson *et al.*, 1969). Although a linear response is claimed by the authors, several disadvantages are apparent. Such disadvantages include: (1) high standard deviation within treated groups, necessitating a low sensitivity; (2) the reality that several tissues, aside from intestine, influence plasma ^{45}Ca concentration (*e.g.*, a new metabolite could give an increased plasma ^{45}Ca by having its effect on bone or kidney rather than intestine itself). If stimulation of calcium absorption is the desired effect then measurement of calcium movement from the gut *per se* should be desirable. Such a method, using the duodenum *in situ*, was employed with success during this study. The activity of 1,25-DHCC appeared equivalent to 25-HCC in stimulation of calcium absorption when assayed 24 hr following dosage. Previous studies employing the 24-hr time period but using plasma ^{45}Ca for estimation of absorption, have revealed values ranging from 1 to 3.5 that obtained using 25-HCC (Myrtle *et al.*, 1970; Kodicek *et al.*, 1970). In a more recent study (Myrtle and Norman, 1971), a time-course profile for 1,25-DHCC was obtained wherein its activity was equivalent to 25-HCC at 24 hr, consistent with this report. Inconsistent measurements of the biological activity of 1,25-DHCC may well stem from the method used for measuring calcium absorption or the procedure for isolation of the metabolite.

A most revealing observation of 1,25-DHCC, however, concerns the rapid onset and decline of the metabolites' action in the intestine (Figure 6). The maximal response of 1,25-DHCC is elicited at approximately 10 hr, compared to 24 hr or longer for 25-HCC. This time for maximal response is in agreement with other laboratories (Haussler *et al.*, 1971; Myrtle and Norman, 1971).

A shorter response time than that for 25-HCC combined with studies using cycloheximide and actinomycin D (Tanaka and DeLuca, 1971; Gray and DeLuca, 1971) suggest that 1,25-DHCC is the metabolically active form of vitamin D_3, at least in the intestine. Although 1,25-DHCC acts in the intestine, recent results suggest that it is snythesized in the kidney (Fraser and Kodicek, 1970; Gray *et al.*, 1971) and then sequestered by the target tissue.

An attempt to quantitatively compare the total activity of 1,25-DHCC to that of 25-HCC or of vitamin D_3 in the stimulation of calcium absorption seems rather tenuous at this time. However, integration of areas under the time-course profiles could give a reasonable estimate to the biological potency of vitamin D and its metabolites, but has not yet been carried out to the authors' knowledge. Vitamin D repletion of rachitic rats promotes calcification in the epiphysis which can be quantitated. This is the basis of the sensitive rat-line test used for vitamin D and related compounds. This assay is based on the entire effect of the administered substance and thereby measures the total effect of the vitamin or metabolite in in-

[3] The term peak V is used in this communication as regards to a polar metabolite of vitamin D_3 found in intestine after a physiological dose of either vitamin D_3 or 25-HCC. Other laboratories use a different notation for the same metabolite (Haussler *et al.* (1968) used 4b and Lawson *et al.* (1969) used P). This polar metabolite has recently been identified as 1,25-DHCC (Holick *et al.*, 1971a,b).

ducing calcification. Although 1,25-DHCC acts much more rapidly in stimulating intestinal calcium transport and gives a larger maximal response than 25-HCC, it is obviously of short duration. 25-HCC and vitamin D_3, on the other hand, are of long duration. The cumulative effect of the 1,25-DHCC is probably less than either vitamin D_3 or 25-HCC. This possibility is reflected in the line test result which revealed 1,25-DHCC to be only 0.4 as active as 25-HCC.

Kodicek *et al.* (1970) have reported no change in plasma calcium concentration 24 hr following 25-HCC or 1,25-DHCC administration (325 pmoles). In contrast, previously mentioned results (Figure 7) show that 1,25-DHCC is active in bone mineral mobilization, although somewhat less effective than 25-HCC. Experiments presently in progress may well provide the necessary information to allow a deduction as to which is the metabolically active form of vitamin D in bone mineral mobilization.

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