Biochemistry

Volume 9, Number 19 September 15, 1970

Metabolism of 25-Hydroxycholecalciferol in Target and Nontarget Tissues*

Robert J. Cousins,† Hector F. DeLuca,‡ and Richard W. Gray

ABSTRACT: The *in vivo* metabolism of 25-hydroxycholecalciferol (25-HCC), a biologically active metabolite of vitamin D_3, was studied in the bone, intestinal mucosal cellnuclei, kidney, liver, and plasma of vitamin D deficient rats. The intestinal nuclei within minutes convert 25-HCC into two metabolites, peaks VI and V, both more polar chromatographically than the parent compound. The kinetics of appearance of these metabolites in mucosal nuclei indicates peak VI is the precursor of peak V. In bone the peak VI intermediate is not detected as the relative amount of peak V increases with time. A substantial ability to metabolize 25-HCC is observed in the liver and kidney. However the plasma maintains a low circulatory level of these metabolites. The rapid metabolic conversion of 25-HCC in target tissues, *i.e.*, bone and intestine, well before any observable physiological effect supports the hypothesis that 25-HCC is the circulating or hormonal form and peaks V and/or VI are tissue-generated regulatory forms of vitamin D_3.

An *in vivo* effect of physiological doses of vitamin D on the mobilization of bone mineral was conclusively established by Carlsson (1952). However, a direct *in vitro* vitamin D effect on bone resorption has never been conclusively established. Recently Trummel *et al.* (1969) were able to show a marked positive *in vitro* effect on bone resorption with 25-hydroxycholecalciferol (25-HCC),[1] the circulating active form of vitamin D_3 produced enzymatically in the liver (Ponchon and DeLuca, 1969a). Similarly it has been demonstrated that 25-HCC has a direct *in vitro* effect on the calcium-transport system of the small intestine while vitamin D_3 does not (Olson and DeLuca, 1969). Clearly then vitamin D_3 must first be converted into an active form (25-HCC) before its physiological expression is possible. Therefore functional metabolism of vitamin D_3 is best studied at the tissue level with 25-HCC rather than with the dietary form of the vitamin.

Recently Cousins *et al.* (1970) have shown that tritium labeled 25-HCC is rapidly converted in the mucosa of the small intestine into two metabolites both more polar chromatographically than either vitamin D or 25-HCC. The formation of these two metabolites, referred to as peaks V and VI, is rapid enough to be of great importance in understanding the mode of 25-HCC action on intestinal calcium transport. The appearance of both peaks V and VI occurs well before the pulse labeling of intestinal nuclear RNA in response to 25-HCC; hence the hypothesis was advanced that further metabolism of 25-HCC could be necessary within vitamin D target tissues, *i.e.*, intestinal mucosa, bone, and kidney, prior to physiological action. Therefore, 25-HCC could be considered a circulating or hormonal form whereas peak V and/or peak VI could be tissue active forms of vitamin D.

Since 25-HCC has a direct effect on calcium mobilization from bone as well as calcium absorption by the intestine an analogous metabolic sequence might be expected in bone as in mucosal cells. The present experiments compare the *in vivo* metabolism of [^{3}H]-25-HCC in bone, liver, intestine, and kidney with the circulating metabolites present in the plasma.

Materials and Methods

Animals. All experiments were conducted with male, albino rats obtained when 20 days old (Holtzman Co., Madison, Wis.). The animals were maintained individually in hanging

* From the Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706. *Received April 30, 1970.* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by Grant AMO-5800-08 from the U. S. Public Health Service and by the Wisconsin Alumni Research Foundation.

† Recipient of U. S. Public Health Service Postdoctoral Fellowship No. 5 F02 AM 41810-02.

‡ To whom all inquiries should be addressed.

[1] Abbreviation used is: 25-HCC, 25-hydroxycholecalciferol, 25-OH vitamin D_3.

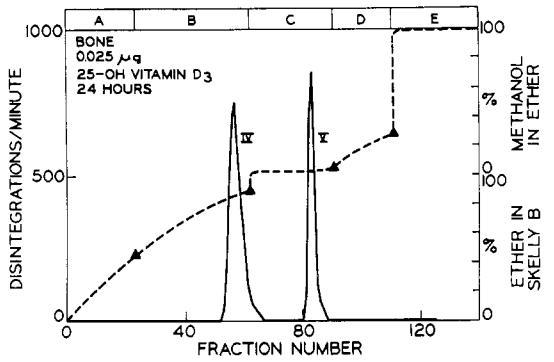

FIGURE 1: Silicic acid chromatography of lipid extract of bone 24 hr after 0.025 μg of [^{3}H]-25-hydroxycholecalciferol. (———) Radioactivity. (------) Gradient. The letters A to E refer to the solvent changes and elution gradients described in the text.

wire cages and were allowed food and distilled water *ad libitum*. The purified vitamin D deficient diet (Guroff *et al.*, 1963) fed throughout the experimental period contained 0.47% calcium and 0.3% phosphorus. Fat-soluble vitamins A, E, and K, dissolved in Wesson Oil, were placed on top of fresh diet three times per week. After 5 weeks on this diet the rats were vitamin D deficient and were used for experiments.

Isotope. [26,27-^{3}H]-25-Hydroxycholecalciferol, with a specific activity of 1.3 Ci/mmole (T. Suda and H. F. DeLuca, in preparation), was synthesized in this laboratory. The radiochemical purity of this preparation was routinely checked by silicic acid chromatography (Ponchon and DeLuca, 1969b).

Preparation of Tissues. Thirty vitamin D deficient rats were divided into five equal groups, each group being sacrificed at one of the time periods reported. After the animals had been fasted for 15 hr each was placed under light ether anesthesia and 0.025 μg (1.4 IU) of the isotopically labeled 25-HCC was administered intrajugularly in 25 μl of 95% ethanol. The rats were killed by exanguination and the blood was collected in centrifuge tubes containing two drops of heparin. The peritoneal cavity was then quickly opened, and the liver and both kidneys were removed, washed in ice-cold 0.9% NaCl, blotted dry, and weighed. The first 48 cm of the duodenal end of the small intestine was removed and a crude nuclear fraction of the mucosal layers was prepared as previously reported (Cousins *et al.*, 1970). Tissue samples not immediately extracted were held at −20°. Both hind legs were dissected to remove adhering muscle, skin, and connective tissue from the femoral and tibial bones. After the bones were split lengthwise and the marrow carefully removed each half was placed on an aluminum foil sheet which rested on solid CO_2. The bone sections were allowed to cool for 30–60 min and were then pulverized with an equal volume of solid CO_2 in a stainless steel mortar and pestle maintained at a solid CO_2–ethanol temperature.

Lipid Extraction. The frozen, pulverized bone was immediately mixed with 150 ml of methanol–chloroform (2:1, v/v and stirred for 24 hr at 4°. The bone fragments were subsequently removed by filtration through glass wool, then 50 ml of chloroform and 90 ml of distilled water were added to produce a two-phase system (Lund and DeLuca, 1966).

Twenty-five to thirty per cent homogenates of minced kidney and liver samples were prepared in 0.25 M sucrose. Two volumes of methanol and one volume of chloroform were subsequently added to an eight-tenth part of homogenate, then the mixture was shaken vigorously for 2 min and allowed to stand for 1 hr at room temperature. The phases were separated overnight at 4° by addition of one part of chloroform. Plasma obtained from the heparinized blood and suspensions of crude nuclei from intestinal mucosal cells were extracted in an identical manner.

Chloroform layers containing the 25-HCC metabolites were removed and evaporated to dryness on a flash evaporator. When necessary, residual water in this phase was removed as an azeotrope by addition of 100% ethanol near the end of the drying step. The final lipid extract was dissolved in 0.2 ml of chloroform and 5 ml of Skelly B (predominately *n*-hexane; bp 67–68°) for chromatography.

Chromatography. Silicic acid chromatography of the lipid extracts followed a five-step hyperbolic gradient system specifically designed for the separation and quantitation of polar metabolites of vitamin D (Ponchon and DeLuca, 1969b). The column (1 × 55 cm) consisted of heat-activated silicic acid slurried in Skelly B. Exponential gradients of ether and methanol were produced by sequential addition of 250 ml of 75% (v/v) ether in Skelly B, 400 ml of 100% ether, 300 ml of 5% (v/v) methanol in ether, and 200 ml of 50% (v/v) methanol in ether to the holding chamber of the gradient generating apparatus. The constant-volume mixing chamber originally contained 230 ml of Skelly B. The terminal eluate was 200 ml of 100% methanol applied directly to the column. The gradients produced with this system are referred to as A, B, C, D, and E, respectively, in Figure 1. Ten-milliliter fractions were collected directly in scintillation counting vials.

Radioactivity Determination. The eluate of each fraction was evaporated under forced air and 15 ml of a toluene-counting solution was added (Herberg, 1960). The tritium content was measured with a Packard Tri-Carb liquid scintillation spectrometer, Model No. 3003 (Packard Instrument Corp., LaGrange, Ill.), equipped with an external standardization system. Counting efficiencies were 30–35% for all samples.

Results

A typical profile of the silicic acid chromatography of a lipid extract of rat bone 24 hr after a 0.025-μg dose of [^{3}H]-25-HCC is shown in Figure 1. Only two metabolites are evident, *i.e.*, peak IV (25-HCC), the presursor metabolite, and peak V, the product metabolite which is eluted with the 5% methanol in ether gradient. Polar metabolites of vitamin D and 25-HCC have been detected previously in bone (Lund and DeLuca, 1966; Kodicek, 1970; Lawson *et al.*, 1969), but an extended chromatographic system capable of detecting closely related polar compounds was not employed until recently (Ponchon and DeLuca, 1969b). In the present studies the relative amount of peak V in bone appears to increase with time as 25-HCC, the precursor, decreases as demonstrated by the data presented in Figure 2. At none of the time periods examined were any metabolites of greater polarity than peak V eluted from the column. A fraction of low polarity, eluted by the 75% ether in Skelly B gradient, probably an ester or other conjugate of 25-HCC, appears by 5-hr postinjection but has decreased by 24 hr after administration of the dose. The significance of this conjugated form, if any, has not been studied; however Lund and DeLuca (1966) reported 10% of

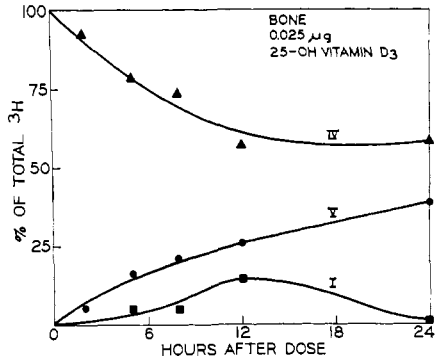

FIGURE 2: Conversion of [^{3}H]-25-hydroxycholecalciferol (IV) into peak I and peak V in bone. The data are expressed as per cent of chromatographed radioactivity.

TABLE I: Distribution of ^{3}H-Containing Metabolites in Kidney Following a Dose of [^{3}H]-25-Hydroxycholecalciferol.[a]

Metabolite (Peak)	Hr after Dose		
	2	12	24
IV	80.8	56.2	54.2
V	16.2	30.6	45.8
VI	3.0	13.2	0

[a] Three groups of six vitamin D deficient rats each were injected, intrajugularly, with 0.025 μg of [^{3}H]-25-HCC. One group was sacrificed at each time period reported. The kidney samples within each group were pooled. Data are expressed as percentage of total ^{3}H eluted from the column.

the metabolites in bone extracts 24 hr after a 500-IU dose of vitamin D_3 was a metabolite of lower polarity than the vitamin. Subsequent work (Lund *et al.*, 1967) demonstrated this metabolite to be an ester of vitamin D_3. Since an esterifying mechanism is probably present in bone it undoubtedly utilizes 25-HCC as well as vitamin D_3 as a substrate.

The time course of appearance of polar metabolites in the nuclear fraction of intestinal mucosa is shown in Figure 3. The relative amount of 25-HCC decreases rapidly in this fraction concomitant with a rapid increase in peak VI, a metabolite eluted from the column by the 50% methanol in ether gradient. Two hours after the dose, peak VI begins to decrease whereupon peak V becomes a major metabolite. The transient nature of peak VI suggests it is an intermediate between 25-HCC and peak V.

The kidney appears to have a slightly higher ability than bone to metabolize 25-HCC (Table I) but within the time sequence examined this ability is clearly less than seen in intestinal nuclei. The amount of a peak VI metabolite in this organ, although small, suggests that peak V production occurs *via* a pathway similar to the mucosal system wherein peak VI is an intermediate. While a primary role of vitamin D on the renal reabsorption of calcium and phosphate is debated it nevertheless is interesting that 25-HCC is rapidly metabolized in the kidney as in the established primary target organs of vitamin D_3 action (DeLuca, 1967).

The distribution of ^{3}H-containing metabolites in the plasma is shown in Table II. The major metabolite circulating in the plasma 2–24 hr after the dose is clearly unmetabolized 25-HCC. The lag in the appearance of polar metabolites in plasma compared to the intestine, bone, and kidney is apparent however.

The distribution of metabolites in the liver extracts (Table III) demonstrates that this organ also has the ability to metabolize 25-HCC. The rate of 25-HCC conversion in liver appears to be similar to that of bone and kidney but is clearly less rapid than in the intestine.

Discussion

The metabolism of 25-HCC by bone as demonstrated in this report appears to be considerably slower than in intestinal mucosa. Active metabolism is detected in the latter within 7.5 min after a 0.025-μg dose (Cousins *et al.*, 1970) while 2–5 hr are necessary before 25-HCC conversion is apparent in bone. This dichotomy however might be expected if the function of 25-HCC in these two tissues is different. Calcium absorption occurs within the mucosa after a 3–4-hr lag period following administration of 0.25 μg of 25-HCC to a

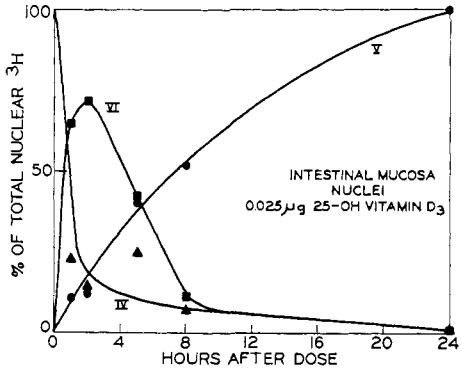

FIGURE 3: Production of metabolites in intestinal mucosal nuclei following administration of [^{3}H]-25-hydroxycholecalciferol. The data are expressed as per cent chromatographed radioactivity. The designation IV represents 25-HCC while V and VI represent the polar unidentified metabolites.

TABLE II: Distribution of ^{3}H-Containing Metabolites in Plasma Following a Dose of [^{3}H]-25-Hydroxycholecalciferol.[a]

Metabolite (Peak)	Hr after Dose				
	2	5	8	12	24
IV	94.9	87.5	82.5	78.1	71.4
V	5.1	12.5	17.5	21.9	28.6

[a] Five groups of six vitamin D deficient rats each were administered, intrajugularly, 0.025 μg of [^{3}H]-25-HCC. One of the groups was then sacrificed at each time period reported and the plasma pooled. Data are expressed as percentage of total ^{3}H eluted from the column.

TABLE III: Distribution of ^{3}H-Containing Metabolites in Liver Following a Dose of [^{3}H]-25-Hydroxycholecalciferol.[a]

Metabolite (Peak)	Hr after Dose				
	2	5	8	12	24
IV	80.3	66.4	62.2	47.4	45.7
V	14.9	27.5	31.6	47.2	46.7
VI	4.8	6.1	6.2	5.4	7.6

[a] Five groups of six vitamin D deficient rats each were administered, intrajugularly, 0.025 μg of [^{3}H]-25-HCC. One of the groups was then sacrificed at each time period reported. The liver samples within each group were pooled. Data are expressed as percentage of total ^{3}H eluted from the column.

vitamin D deficient rat (Blunt *et al.*, 1968). During this lag period substantial 25-HCC metabolism and a stimulation of nuclear RNA synthesis have occurred. The necessity for 25-HCC transformation into another metabolically active compound, perhaps peak V or VI, could explain the 1-hr lag in a detectable increase in nuclear RNA synthesis observed in this target tissue. Similarly an increase in serum calcium levels of vitamin D deficient rats maintained on a low calcium diet starts 4–8 hr after a larger dose of 25-HCC reaching a maximal response by 12 hr (Blunt *et al.*, 1968). This lag in bone mobilization therefore could likewise be explained in part by a necessity for further metabolism of 25-HCC before a physiological expression as had been postulated in mucosa. As was suggested, however, as an alternative hypothesis these polar compounds could limit or shut down the initial event at the genone level triggered by 25-HCC (Cousins *et al.*, 1970).

Metabolism of 25-HCC in the kidney also occurs within 2 hr. Moreover a peak VI metabolite is evident at two of the time periods investigated. While vitamin D induced renal calcium and phosphate reabsorption is an open question, substantial 25-HCC metabolism in the kidney is clear. Hence since 25-HCC conversion occurs in two primary targets of vitamin D, bone and intestine, the kidney could possibly also be a primary target organ. It is interesting to note in contrast that in the muscle tissue surrounding the femur, 90% of tritium-containing metabolites present is 25-HCC (R. J. Cousins and H. F. DeLuca, unpublished results) by 8 hr after the dose.

The later appearance of metabolites in the plasma strongly suggest that these metabolites arise at least partially in target tissues. Since the plasma shows the appearance of polar 25-HCC metabolites only well after the physiological action of 25-HCC in bone and intestine is well in progress, it appears these metabolites originate in the target organs and enter the systemic circulation and not *vice versa*. Evidence for this fact was recently demonstrated in a detailed study of the subcellular *in vivo* 25-HCC metabolism in mucosa of rats that had received 0.25 μg of [^{3}H]-25-HCC (Cousins *et al.*, 1970). While the plasma and intestinal mucosal cytoplasm contained 92 and 87% unconverted 25-HCC, respectively, the mucosal nuclear extracts contain only 27% 25-HCC 60 min after the dose. Moreover, conversion of 25-HCC into peaks V and VI has been demonstrated in intestinal homogenates *in vitro* (R. W. Gray, R. J. Cousins, and H. F. DeLuca, unpublished results).

Of great interest was the recent separation of peak V from the plasma of chicks and hogs into three major separate compounds (Suda *et al.*, 1970). The least polar of these compounds was conclusively identified as 21,25-dihydroxycholecalciferol and was found to be very biologically active in the mobilization of bone mineral while having a slight effect on intestinal calcium absorption. It is therefore tempting to suggest from these data that the peak V metabolite found in bone, which has been shown to be homogeneous with three different chromatographic systems (silicic acid, Celite partition, and gel filtration), is hydroxylated at position C-21 subsequent to the administration of 25-HCC. The other plasma peak V compounds could originate therefore in other tissues. This hypothesis is currently under investigation.

While the significance of the further metabolism of 25-HCC is still unknown we can now state that metabolites are formed in primary targets of vitamin D. This metabolism does not appear to be specifically limited to these tissues, but appears to be most rapid in the intestine. The data presented in this report support the hypothesis stated previously that 25-HCC is the circulating or hormonal form and peak V is a tissue-generated form which probably has a specific regulatory function *in situ*.

References

Blunt, J. W., Tanaka, Y., and DeLuca, H. F. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 717.

Carlsson, A. (1952), *Acta Physiol. Scand. 26*, 212.

Cousins, R. J., DeLuca, H. F., Suda, T., Chen, T., and Tanaka, Y. (1970), *Biochemistry 9*, 1453.

DeLuca, H. F. (1967), *Vitam. Horm. (New York) 25*, 315.

Guroff, G., DeLuca, H. F., and Steenbock, H. (1963), *Amer. J. Physiol. 204*, 833.

Herberg, R. J. (1960), *Anal. Chem. 32*, 42.

Kodicek, E. (1970), *in* Fat Soluble Vitamins, DeLuca, H. F., and Suttie, J. W., Ed., Madison, Wis., University of Wisconsin Press (in press).

Lawson, D. E. M., Wilson, P. W., and Kodicek, E. (1969), *Nature (London) 222*, 171.

Lund, J., and DeLuca, H. F. (1966), *J. Lipid Res. 7*, 739.

Lund, J., DeLuca, H. F., and Horsting, M. (1967), *Arch. Biochem. Biophys. 120*, 513.

Olson, E. B., and DeLuca, H. F. (1969), *Science 165*, 405.

Ponchon, G., and DeLuca, H. F. (1969a), *J. Clin. Invest. 48*, 1273.

Ponchon, G., and DeLuca, H. F. (1969b), *J. Nutr. 99*, 157.

Suda, T., DeLuca, H. F., Schnoes, H. K., Ponchon, G., Tanaka, Y., and Holick, M. (1970), *Biochemistry 9*, 2917.

Trummel, C., Raisz, L. G., Blunt, J. W., and DeLuca, H. F. (1969), *Science 163*, 1450.