In the case where χ is bound to a site on the protomer different from both the ω and the ψ sites, a, b, and a/b may all change and

$$a_\chi = a_0\frac{1 + \epsilon\chi}{1 + \zeta\chi}$$

$$b_\chi = b_0\frac{1 + \eta\chi}{1 + \zeta\chi}$$

where

$$\eta = \frac{\sum_j L_1^j B_\psi^j B_\omega^j B_\chi^j}{\sum_j L_1^j B_\omega^j}$$

References

Buc, H. (1967), *Biochem. Biophys. Res. Commun. 28*, 59.

Buc, M. H., and Buc, H. (1967), *in* Regulation of Enzyme Activity and Allosteric Interactions, Kvamme, E., and Pihl, A., Ed., London, Academic Press, p 109 (4th Meeting, FEBS Symp., Oslo 1967).

Cleland, W. W. (1967), *Ann. Rev. Biochem. 36*, 77.

Koshland, D. E., Nemethy, G., and Filmer, D. (1966), *Biochemistry 5*, 365.

Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.

Whitehead, E. P. (1970a), *Progr. Biophys. Mol. Biol. 21*, 321.

Whitehead, E. P. (1970b), Document No. NAPS-00885 (see title footnote).

Metabolism and Subcellular Location of 25-Hydroxycholecalciferol in Intestinal Mucosa*

R. J. Cousins,† H. F. DeLuca,‡ T. Suda, T. Chen, and Y. Tanaka

ABSTRACT: The metabolism and subcellular distribution of 25-hydroxycholecalciferol, a biologically active metabolite of cholecalciferol (vitamin D_3) was investigated in the intestinal mucosa of vitamin D deficient rats. The mucosal nuclear fraction accumulates approximately 50 and 25% of the total cellular ^3H after administration of 0.025 and 0.25 μg of [26,27-^{3}H]-25-hydroxycholecalciferol, respectively. Both 1,2-^3H- and 26,27-^3H-labeled 25-hydroxycholecalciferol are converted *inv ivo* by the intestinal mucosa into polar metabolites, referred to as peak V and peak VI. Within 30 min after a 0.025-μg dose of [26,27-^{3}H]-25-hydroxycholecalciferol, 72% of the nuclear radioactivity is peak VI, the more polar of the two metabolites. Two hours after the dose the relative amount of peak VI steadily decreases and after 8 hr peak V has increased to 58% of the total tritium-containing metabolites in the nuclear fraction. The time course of appearance of these metabolites indicates peak VI is the probable precursor of peak V. The paucity of these polar metabolites in the cytoplasmic fractions suggest they are of nuclear origin and hence may be of significance in understanding at the nuclear level the mode of action of vitamin D on the calcium transport mechanism in the small intestine.

The currently accepted model for vitamin D action in the small intestine proposes that specific genetic information is unmasked in response to the vitamin, leading ultimately to the synthesis of specific functional protein(s) involved in a calcium transport system (DeLuca, 1967). A response in calcium absorption is known to lag several hours after administration of vitamin D and recent evidence partially explains the reason for this lag period. First it has been conclusively established that vitamin D_3 (cholecalciferol) must be converted enzymatically by the liver into an "active form" (Ponchon and DeLuca, 1969a; Horsting and DeLuca, 1969) which has been identified as 25-hydroxycholecalciferol (Blunt *et al.*, 1968a).[1] The next important event in vitamin D action occurs at or in the nucleus of the intestinal mucosal cell where the vitamin and its metabolites accumulate (Stohs and DeLuca, 1967). Then by an unknown mechanism, a metabolite of vitamin D stimulates the synthesis of nuclear RNA, as demonstrated by the increased incorporation of [^{3}H]orotic acid into nuclear RNA (Stohs *et al.*, 1967) as well as the increased DNA template activity of these cells (Hallick and DeLuca, 1969). Presumably this nuclear RNA includes specific mRNA which codes for the protein components of the calcium transport system. Both of these nuclear responses occur well before any detectable response in the calcium transport mechanism (Blunt *et al.*, 1968b; Wasserman and Taylor, 1968), but undoubtedly RNA synthesis adds substantially to the lag in vitamin D action.

* From the Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706. *Received November 14, 1969.* 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.

† Recipient of U. S. Public Health Service Postdoctoral Fellowship No. 7-F02-AM-41, 810-01A1.

‡ To whom all inquiries should be addressed.

[1] This compound may also be referred to as 25-OH vitamin D_3, 25-HCC, or peak IV.

Recent studies on the metabolism of [^{3}H]vitamin D_3 have demonstrated that other metabolites, more polar chromatographically than either cholecalciferol or 25-hydroxycholecalciferol, are present in certain tissues and blood following administration of physiological doses of the radioactive vitamin (Ponchon and DeLuca, 1969a,b; Haussler *et al.*, 1968; Lawson *et al.*, 1969). However it has not been established if these metabolites are produced early enough to be of importance in understanding the lag in vitamin D response. Hence in order to evaluate the biological significance of these polar metabolites a new approach had to be taken. The production of the "active form" of vitamin D is undoubtedly closely regulated *in vivo* to facilitate calcium homeostasis, therefore it is obvious that meaningful studies of vitamin D metabolism in target tissues must be carried out at the level of the active form rather than the dietary form of the vitamin. The synthesis of high specific activity [^{3}H]-25-hydroxycholecalciferol has provided the tool necessary to follow this new approach.

The studies described in this paper were undertaken to establish that the polar metabolites of vitamin D_3 actually originate *in vivo* from the active form, to identify the subcellular location and origin of these metabolites as well as to analyze the sequence of their appearance in the intestinal mucosa following intravenous administration of physiological doses of [^{3}H]-25-hydroxycholecalciferol.

Experimental Procedure

Animals. Male rats (Holtzman Co., Madison, Wis.) obtained at 20 days of age were maintained individually in hanging wire cages and given food and distilled water *ad libitum*. The purified diet (Guroff *et al.*, 1963) fed throughout these experiments contained 0.47% calcium and 0.3% phosphorus. Vitamins A, E, and K in Wesson oil were placed on top of fresh diet three times a week. After 4 weeks on this diet the rats were vitamin D deficient by the criteria of lowered serum calcium levels and retarded growth.

Isotopes. [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. [1,2-^{3}H]-25-Hydroxycholecalciferol with a specific activity of 650 mCi/mmole was prepared from the plasma of chicks previously administered [1,2-^{3}H]cholecalciferol (Neville and DeLuca, 1966). Radiochemical purity of these preparations was routinely checked by silicic acid chromatography (Ponchon and DeLuca, 1969b).

The [5-^{3}H]orotic acid (New England Nuclear Corp., Boston, Mass.) used in the RNA pulse-labeling experiments had a specific activity of either 4.1 or 940 mCi per mmole.

Isotope Administration and Mucosa Preparation. All animals were fasted for 12 to 15 hr prior to sacrifice. After placing the animals under light ether anesthesia the specific isotopically labeled compound was administered intrajugularly in 0.02 ml of 95% ethanol, at one of the time intervals before sacrifice. The rats were killed by a sharp blow on the head, the peritoneal cavity was quickly opened, and 48 cm of the duodenal end of the small intestine was removed and placed in ice-cold 0.25 M sucrose–TKM buffer (pH 7.4).[2] The entire small intestine was removed for extraction in the whole tissue experiments. All intestinal segments were freed of fat and mesentery, then slit open, washed in fresh buffer, blotted on absorbant paper, and placed mucosal side up on an ice-cold stainless steel plate. The mucosa was then scraped from the serosa and submucosal muscle layers with a glass slide (Crane and Mandelstam, 1960).

Tissue Fractionation. Mucosal scrapings from six rats were weighed and a 10% homogenate was prepared in 0.25 M sucrose–TKM buffer utilizing fifteen strokes of a motor-driven glass–Teflon Potter–Elvehjem homogenizer (A. H. Thomas Co., Philadelphia, Pa.) operated at 800 rpm. The subcellular fractionation closely followed the procedure of Schneider and Hodgeboom (1950). The crude nuclear fraction was obtained by centrifugation at 600*g* for 10 min in a refrigerated Lourdes centrifuge equipped with a 9-RA rotor. After washing the nuclear pellet twice, the postnuclear supernatant was centrifuged at 7500*g* for 10 min to obtain the mitochondrial fraction. The mitochondrial pellet was washed once. The 7500*g* supernatant was then centrifuged at 105,000*g* for 60 min in a Spinco Model L-2 ultracentrifuge employing a No. 50 titanium rotor to obtain the microsomal (pellet) and cytoplasmic (supernatant) fractions. All fractionation and centrifugation steps were conducted at 0–4°. Each subcellular fraction was subsequently resuspended in an appropriate amount of 0.25 M sucrose–TKM buffer. Subcellular suspensions not immediately extracted were held at −20°. These operations were carried out individually in the experiments involving the 0.25-μg doses and on pooled mucosa in the experiments involving the 0.025-μg doses. In the 0.25-μg experiments after appropriate aliquots of the six nuclear suspensions and supernatants were removed the remainder of each respective fraction was combined and subsequently extracted as described below.

Extraction and Chromatography of Vitamin D Metabolites. The radioactive nuclear suspensions and supernatants were extracted by the chloroform–methanol procedure described by Lund and DeLuca (1966). Two parts of methanol and one part of chloroform were added to eight-tenths part of either a nuclear suspension or 105,000*g* supernatant. This one-phase system was shaken vigorously for 2 min and then allowed to stand for 30 min at room temperature. A two-phase system was produced by addition of one part chloroform and then allowing the mixture to stand overnight in a cold room. The chloroform layer was then removed and evaporated to dryness on a flash evaporator. Residual water in this phase was removed as an azeotrope by repeated additions of 100% ethanol at the end of the drying step. The final lipid extract was dissolved in Skellysolve B (predominately *n*-hexane; bp 67–68°) for chromatography.

Silicic acid chromatography of the lipid extracts was carried out according to the procedure described in detail by Ponchon and DeLuca (1969b). Columns (1 × 55 cm) of activated silicic acid were prepared in Skellysolve B. The ^{3}H-containing samples were eluted from the columns by sequential additions of 250 ml of 75% (v/v) diethyl ether in Skellysolve B, 400 ml of 100% diethyl ether, 300 ml of 5% (v/v) methanol in diethyl ether, and 200 ml of 50% (v/v) methanol in diethyl ether to the holding chamber of an exponential gradient generating apparatus. The constant volume mixing chamber initially contained 230 ml of Skellysolve B. The columns were stripped of metabolites with 200

[2] Abbreviation used is: TKM, 0.05 M Tris-HCl–5 mM $MgCl_2$–0.025 M KCl.

TABLE I: Effect of 0.25 μg of 25-Hydroxycholecalciferol on the Incorporation of [^{3}H]Orotic Acid into Nuclear RNA of Rat Intestinal Mucosa.[a]

Experiment	Time (hr)	Specific Activity of [^{3}H]RNA (dpm/OD) 25-HCC	Control
1	0.5	149 ± 32[b]	150 ± 45[b]
2	1	157 ± 30	119 ± 28
3	2	242 ± 17	140 ± 4
4	4	244 ± 11	204 ± 16

[a] Vitamin D deficient rats were administered 0.25 μg of 25-hydroxycholecalciferol intravenously and sacrificed 0.5, 1, 2, or 4 hr later. Each rat received 50 μCi of [5-^{3}H]orotic acid intravenously 30 min prior to sacrifice. [b] Standard deviation. *Each experiment was replicated at least four times.*

TABLE II: Percentage Distribution of ^{3}H-Containing Metabolites in Serum after a 0.25-μg Dose of [26,27-^{3}H]-25-Hydroxycholecalciferol.[a]

Metabolite (Peak)	Minutes after Administration of Dose 15	30	60	120	180
IV	69.8	74.7	91.5	88.6	88.9
V	7.9	3.1	3.8	7.1	7.7
VI	10.3	1.8	3.0	4.3	1.9
VII	12.0	20.4	1.7	0	1.5

[a] Six vitamin D deficient rats were each injected, intrajugularly, with 0.25 μg of [26,27-^{3}H]-25-OH vitamin D_3 and each group was sacrificed at the indicated times after dosage. Data are expressed as percentage of total ^{3}H eluted from the silicic acid column.

ml of methanol applied directly to the column at the end of the 50% methanol gradient. The gradients produced by this system are referred to as A, B, C, D, and E, respectively, in the figures below. Fractions (10 ml) were collected from all columns.

RNA Pulse Labeling. Vitamin D deficient rats were fasted 12–15 hr and then 0.25 μg of 25-HCC was administered intrajugularly at the desired time.[3] Before sacrifice (0.5 hr) each rat received 50 μCi of [^{3}H]orotic acid intrajugularly. Both injections were given during light ether anesthesia. Crude nuclei from the pooled mucosa of 2–3 rats in each treatment group were obtained by centrifugation as described above, but utilizing 0.25 M sucrose–0.05 M Tris-HCl buffer (pH 7.4). The RNA was isolated by the hot phenol method as described by Stohs *et al.* (1967). The absorbancy and spectra of isolated RNA samples dissolved in deionized water were obtained with a Beckman Model DB-G recording spectrophotometer.

Radioactivity Determination. Aliquots of homogenates, subcellular fractions, and nuclear RNA were prepared for tritium determination by drying each sample in a counting vial under an infrared lamp. Deionized water (1 drop) and 0.5–2.0 ml of NCS solubilizer (Amersham/Searle Corp., Des Plaines, Ill.) was added to each vial. After incubation at 37° for 24-hr solubilization was complete and 15 ml of toluene counting solution (Herberg, 1960) was added.

Tritium content was measured with a Packard Tri-Carb liquid scintillation spectrometer, Model No. 3000 (Packard Instrument Corp., LaGrange, Ill.) equipped with an external standardization system. Counting efficiencies were approximately 35% for chromatographic samples and 20–25% for samples containing NCS reagent.

Results

The results of a time course study (Table I) reveal that a stimulation of nuclear RNA synthesis occurs in response to a 0.25-μg intrajugular dose of 25-hydroxycholecalciferol. Since this response is maximal by 2 hr after administration it is evident that the dose–response time course of 25-HCC, at the nuclear level, is markedly shorter than with a comparable dose of vitamin D_3.

The distribution of ^{3}H-containing metabolites in the lipid extracts of blood serum from rats intravenously dosed with 0.25 μg of [26,27-^{3}H]-25-hydroxycholecalciferol is shown in Table II. At each time period reported the major component appears to be unchanged 25-HCC (peak IV). The small amounts of peak V (a metabolite(s) eluted with 5% methanol in ether) and peak VI (a metabolite(s) eluted with 50% methanol in ether) in the serum extracts are not artifactual however, since the injected 25-HCC was radiochemically pure. At the earliest time periods, 15 and 30 min, substantial amounts of a peak VII metabolite are eluted from the columns by the 100% methanol gradient. The significance of the rapid appearance and disappearance of peak VII is not known.

TABLE III: Percentage Distribution of ^{3}H-Containing Metabolites in Intestinal Mucosa after an Intrajugular Dose of [^{3}H]-25-Hydroxycholecalciferol.[a]

Label Position	Metabolite (Peak)	Minutes after Administration of Dose 3	15	30	60	180
26,27-^{3}H	IV	82.2	56.7	44.4	57.6	52.7
	V	9.7	9.6	8.2	10.2	25.7
	VI	8.1	33.7	47.4	33.2	21.6
1,2-^{3}H	IV			57.8		
	V			17.6		
	VI			24.6		

[a] Six vitamin D deficient rats were each injected, intrajugularly, with either 0.25 μg of [26,27-^{3}H]-25-OH vitamin D_3 or 0.1 μg of [1,2-^{3}H]-25-OH vitamin D_3 and each group was sacrificed at the indicated times. Data are expressed as percentage of total ^{3}H eluted from the column.

[3] The nonradioactive 25-HCC was kindly supplied in part by the Upjohn Co., Kalamazoo, Mich., and the Philips-Duphar Co., Weesp, The Netherlands.

TABLE IV: Subcellular Distribution of ^3H in Intestinal Mucosa after a 0.25-μg Dose of [^3H]-25-Hydroxycholecalciferol.[a]

Fraction	Minutes after Administration of Dose			
	15	30	60	120
Crude nuclei	26.9 ± 2.2[b]	31.5 ± 4.3	32.2 ± 5.2	21.7 ± 3.5
Mitochondria	6.3 ± 0.8	6.3 ± 1.2	5.5 ± 1.8	5.6 ± 1.5
Microsomes	10.9 ± 1.3	9.7 ± 2.3	9.4 ± 1.8	9.9 ± 2.2
Supernatant	52.5 ± 3.7	51.0 ± 7.8	52.3 ± 5.5	58.9 ± 2.7
Average total mucosal dpm recovered per animal	12,853	19,074	19,474	19,883

[a] Six vitamin D deficient rats were each injected, intrajugularly, with 0.25 μg of [26,27-^3H]-25-OH vitamin D_3 and each group was sacrificed at the indicated times. Data are expressed as percentage of total ^3H recovered. [b] Standard deviation.

The results of silicic acid chromatography of lipid extracts from intestinal mucosal homogenates prepared from vitamin D deficient rats given 0.25 μg of [26,27-^3H]-25-HCC are summarized in Table III. As early as 15 min after the injection, a marked conversion of peak IV (25-HCC) into peak VI has occurred and by 30 min 47% of the chromatographed chloroform-soluble radioactivity is present as peak VI. The relative abundance of peak V rises steadily at 1 hr after the dose concomitant with a decrease in the relative amount of peak VI.

It is apparent that 25-HCC is rapidly converted *in vivo* into polar metabolites. Therefore, to verify these results a 30-min *in vivo* study was carried out with 0.05 μg of [1,2-^3H]-25-HCC. While the design of this experiment was not strictly comparable with the experiments using 26,27-^3H-labeled 25-HCC, the data shown in the lower section of Table III establish that peak V and peak VI metabolites are produced *in vivo* from [1,2-^3H]-25-HCC as well.

The distribution of ^3H within the components of intestinal mucosal cells from vitamin D deficient rats that had received 0.25 μg of [26,27-^3H]-25-HCC is shown in Table IV. The crude nuclear fraction contains 22–32% of the total cellular ^3H while the cytoplasmic fraction (105,000g supernatant) represents at least 51% of the total ^3H. There is a slight decrease in the ^3H content of the nuclei at 120 min and a corresponding ^3H increase in the cytoplasm. The reason for this is not clear at present. Nevertheless, the ^3H content of the nuclei and cytoplasm remain relatively constant throughout the 2-hr period after injection.

The chromatographic profiles of lipid extracts of both the nuclear and cytoplasmic fractions of mucosal cells from vitamin D deficient rats sacrificed 60 min after receiving 0.25 μg of [26,27-^3H]-25-HCC are presented in Figure 1. The relative proportions of peaks IV, V, and VI are 27, 15, and 58% of the total ^3H, respectively, in the nuclei and 87, 5, and 8%, respectively, in the cytoplasm.

A diagrammatic representation of the metabolite distribution in the intestinal mucosa from chromatographic profiles of nuclear and cytoplasmic lipid extracts at 15, 30, 60, and 120 min after administration of 0.25 μg of [26,27-^3H]-25-HCC (peak IV) is shown in Figure 2. Substantial metabolism of 25-HCC is clearly evident in the nuclear fractions as early as 15 min after the dose. Peak VI reaches a maximum level 60 min after administration. The increase in peak VI closely

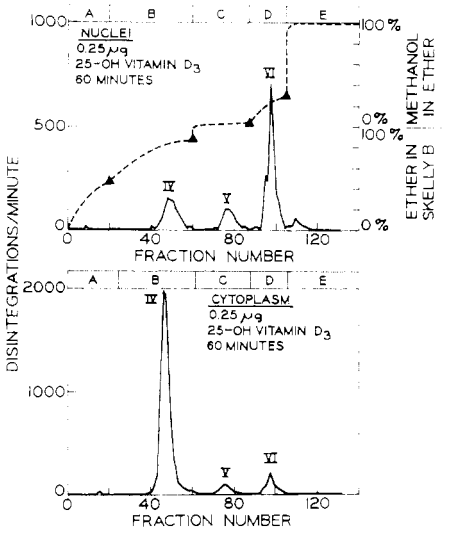

FIGURE 1: Silicic acid chromatography of lipid extracts of intestinal mucosal nuclei and cytoplasm 60 min after 0.25 μg of [26,27-^3H]-25-OH vitamin D_3. (——) Radioactivity; (- - - -) gradient.

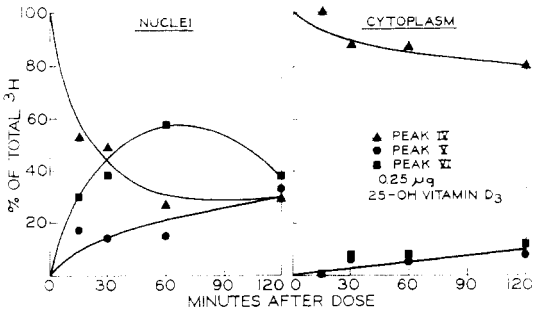

FIGURE 2: Conversion of [26,27-^3H]-25-hydroxycholecalciferol into peak V and peak VI in the nuclei and cytoplasm of intestinal mucosa after a 0.25-μg dose.

TABLE V: Subcellular Distribution of ^3H in Intestinal Mucosa after a 0.025-μg Dose of [^3H]-25-Hydroxycholecalciferol.[a]

Fraction	Minutes after Administration of Dose					
	7.5	15	30	60	120	480
Crude nuclei	56.7	41.8	48.4	41.8	52.6	48.3
Mitochondria	5.4	6.3	6.3	3.8	2.5	2.3
Microsomes	15.5	13.3	14.0	14.6	7.6	6.9
Supernatant	22.4	38.6	31.3	39.8	37.3	42.5
Total dpm recovered	8,504	16,545	17,806	31,960	20,246	11,135

[a] Six vitamin D deficient rats were each injected, intrajugularly, with 0.025 μg of [26,27-^3H]-25-OH vitamin D_3 and each group was sacrificed at the indicated times. Data are expressed as percentage of total ^3H recovered.

coincides with the disappearance of the precursor metabolite, 25-HCC. The relative amount of peak V in the nuclear fraction rises steadily during the time course and since the 25-HCC content remains relatively constant after 60 min it appears plausible that peak V could be derived metabolically from peak VI rather than from 25-HCC. The major metabolite in the cytoplasmic fraction is unchanged 25-HCC. The paucity of metabolites more polar than 25-HCC in the cytoplasm strongly suggest that peaks V and VI are of nuclear origin and not the product of extraintestinal metabolism.

As we have stated above, the circulating level of 25-HCC under normal steady-state conditions is low and the concentration of this metabolite present at intestinal sites at any given time is probably not equivalent to the concentration available to these sites after a 0.25-μg intrajugular dose of this form of vitamin D. Hence to confirm the results obtained with 0.25-μg dose levels, the above subcellular experiments were repeated and expanded employing 0.025-μg intrajugular injections of [^3H]-25-HCC.

In contrast to the 0.25-μg experiments the nuclear and cytoplasmic fractions of mucosal cells accumulate 42–57 and 22–42% of the total cellular ^3H, respectively, at 7.5, 15, and 30 min, and 1, 2, and 8 hr, following 0.025 μg of [26,27-^3H]-25-HCC (Table V).

Profiles of the silicic acid chromatography of lipid extracts from the mucosal cell nuclei of vitamin D deficient rats 0.25, 1, and 8 hr after administration of 0.025 μg of [^3H]-25-HCC are shown in Figure 3. Within 15 min following the injection, 50% of the nuclear radioactivity is in the form of peak VI and 7% as peak V. One hour following the dose, 65% of the total radioactivity is in peak VI, however, by 8 hr after the dose peak V represents the major ^3H-containing metabolite. In addition, substantial amounts of a metabolite or more likely a conjugate of a metabolite, of lower polarity than 25-HCC, has formed by 8 hr after dose administration.

The distribution of ^3H-containing metabolites in the nuclear fraction from the mucosa 7.5, 15, and 30 min, and 1-, 2-, 5-, and 8-hr postadministration of 0.025 μg of 25-HCC is presented diagrammatically in Figure 4. The disappearance of 25-HCC (peak IV) is rapid as peak V and VI accumulate. The marked hyperbolic shape of the peak VI curve helps support the earlier suggestion that peak VI is the precursor of peak V. The cytoplasm contains substantial amounts of the polar metabolites by 8-hr postinjection but not at 2 hr (Table VI).

Discussion

The stimulatory effect of 25-hydroxycholecalciferol on nuclear RNA synthesis (Table I) provides additional support for the hypothesis that the 25-hydroxy metabolite is the active form of vitamin D at the organ level and furthermore demonstrates that it has an effect on the nucleus of intestinal mucosal cells. We are assuming the intestinal mucosa of the vitamin D deficient rat is a fully differentiated tissue and the RNA stimulation observed in the present experiments is largely a reflection of the rapid transcription of that section of the genome which carries the code for the vitamin D sensitive components of the calicum transport system. Since the administration of 25-HCC bypasses the activation mechanism in the liver, the response in RNA synthesis is considerably shorter than that noted by Stohs and DeLuca (1967b) with 10 (0.25 μg) or 2000 (50 μg) IU of vitamin D_3. Blunt *et al.* (1968b) demonstrated that 25-HCC given intravenously initiates calcium transport by everted sacs of rat small intestine 2–3 hr after injection while an equivalent dose of vitamin D_3 requires 8–10 hr to elicit a detectable response. Within this 2–3-hr period, mRNA and protein synthesis must have occurred. Therefore our evidence that a stimulation in RNA occurs within 1 hr and is maximal by 2 hr after a 0.25-μg dose of 25-HCC is consistent with the 3-hr effect on calcium transport in gut sacs.

The present report provides clear evidence that the intestinal

TABLE VI: Distribution of ^3H Metabolites in Intestinal Mucosal Cytoplasmic Fraction after Administration of 0.025 μg of [^3H]-25-Hydroxycholecalciferol.[a]

Metabolite	Hours after Administration	
	2	8
IV	92.0	44.6
V	3.4	37.9
VI	4.6	17.5

[a] Six vitamin D deficient rats were administered 0.025 μg of [26,27-^3H]-25-OH vitamin D_3, intrajugularly, and each group was sacrificed 2 and 8 hr later. Data are expressed as percentage of total radioactivity eluted from the column.

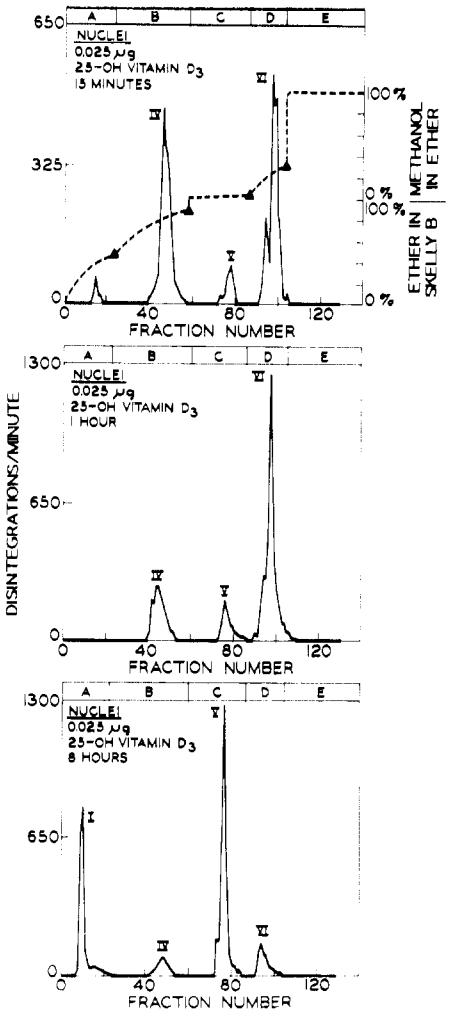

FIGURE 3: Silicic acid chromatography of lipid extract of intestinal mucosal nuclei after 0.025 μg of [26,27-^{3}H]-25-OH vitamin D_3. (——) Radioactivity; (- - - -) gradient.

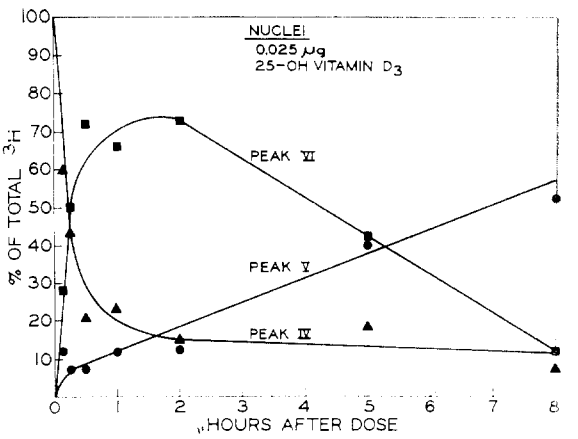

FIGURE 4: Production of metabolites in intestinal mucosal nuclei following intravenous administration of 0.025 μg of [26,27-^{3}H]-25-hydroxycholecalciferol.

mucosal cell nucleus is a probable site of further metabolism of 25-HCC (Figures 2 and 4). The presence of substantial quantities of peaks V and VI in the nuclear fractions and small quantities in the blood and cytoplasmic fractions support this suggestion. It should also be kept in mind that 25-HCC has a direct *in vitro* effect on both bone mobilization (Trummel *et al.*, 1969) and calcium transport (Olson and DeLuca, 1969); therefore meaningful metabolism would have to be at the target tissue level.

From the data at two dose levels this report establishes that [^{3}H]-25-HCC is rapidly converted into peak VI which probably is subsequently converted into peak V. The simultaneous production of peak V and peak VI *via* separate pathways cannot be completely ruled out from our data however. The time course of appearance of these metabolites suggests that peak VI is more likely to be of biological significance, but only future experiments could clarify this point. Stohs and DeLuca (1967) found 80% of the radioactivity from [^{3}H]vitamin D_3 in chick mucosal nuclei in the form of polar metabolites. Similarly Haussler *et al.* (1968) and Lawson *et al.* (1969) have reported the existence of polar metabolites in chick mucosal chromatin and nuclei, respectively. The comparison of our data with data of these investigators must be approached with some reservation. The chick has been shown to rapidly metabolize physiological intravenous doses of vitamin D_3 while in the rat the rate of 25-HCC formation and hence further metabolism is much slower (DeLuca, 1967; G. Ponchon and H. F. DeLuca, 1969, in preparation). Moreover, our experiments were conducted with the active form (25-HCC) administered directly, allowing for the first time evaluation of rapid metabolic conversions otherwise evident only at long periods after vitamin D administration. Lawson *et al.* (1969) report the presence of a tritium deficient polar metabolite (peak P) in the nuclear debris fraction of chick mucosa after 20 IU (0.5 μg) of [1-^{3}H,4-^{14}C]vitamin D_3 were administered. While the chromatographic system employed was not described in detail, peak P was eluted from a silicic acid column with a steep methanol in ether gradient. Therefore this fraction appears to be equivalent to both peak V and peak VI in our chromatographic profiles since a subtle stepwise methanol gradient is necessary to separate these two metabolites. Our data with [1,2-^{3}H]-25-HCC and [26,27-^{3}H]-25-HCC of high specific activity, however, unequivocally establish that peaks V and VI are produced *in vivo* from both specifically labeled compounds.

Recently Kodicek (1969) reported that the radioactivity from 0.375 μg of [1-^{3}H, 4-^{14}C]-25-HCC is largely present as peak P in the crude nuclear fraction by 8 hr after the dose. While the design of the experiment is not strictly comparable with ours and tritium loss in peak P was again observed, it should be noted that if peak V plus peak VI and peak P are essentially equivalent the data are complementary. Kodicek hypothesized on the basis of the tritium loss and the apparent polarity of the fraction that peak P is a ketone derivative of 25-HCC formed *via* an undetected hydroxylated intermediate. It is indeed possible that peak VI is the hydroxylated intermediate and peak V is the ketone derivative postulated by Kodicek. This sequence of reactions would explain the apparent conversion of peak VI into peak V observed in our experiments.

The significance of the possible metabolic conversions of 25-HCC within the intestinal mucosa is not evident at present.

Further conversion may be necessary to establish specificity in target tissue. With such a mechanism, 25-HCC would constitute a "circulating active" form while additional metabolites (peaks V and VI) would constitute "tissue active" forms. This mechanism would explain the specificity and differences in the physiological expression of vitamin D action in bone and intestinal tissues. Bruchovsky and Wilson (1968) reported testosterone is converted by target tissues to additional biologically active metabolites. They have suggested that enzymes which catalyze these further metabolic steps are present only in testosterone sensitive tissues.

An alternative suggestion to explain the significance of further 25-HCC metabolism would be that a regulatory mechanism is necessary within the intestine to limit or shut down the synthesis of calcium transport components prior to mucosal cell destruction within the intestinal lumen. Further work is clearly necessary to establish the validity of the above suggestions or other possible mechanisms before the significance of the intracellular metabolism of 25-hydroxycholecalciferol is fully understood.

References

Blunt, J. W., DeLuca, H. F., and Schnoes, H. K. (1968a), *Biochemistry 7*, 3317.

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

Bruchovsky, N., and Wilson, J. D. (1968), *J. Biol. Chem. 243*, 2012.

Crane, R. K., and Mandelstam, P. (1960), *Biochim. Biophys. Acta 45*, 460.

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

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

Hallick, R. B., and DeLuca, H. F. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 528.

Haussler, M. R., Myrtle, J. F., and Norman, A. W. (1968), *J. Biol. Chem. 243*, 4055.

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

Horsting, M., and DeLuca, H. F. (1969), *Biochem. Biophys. Res. Commun. 36*, 251.

Kodicek, E. (1969), *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 222*, 171.

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

Neville, P. F., and DeLuca, H. F. (1966), *Biochemistry 5*, 2201.

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.

Schneider, W. C., and Hodgeboom, G. H. (1950), *J. Biol. Chem. 183*, 123.

Stohs, S. J., and DeLuca, H. F. (1967), *Biochemistry 6*, 3338.

Stohs, S. J., Zull, J. E., and DeLuca, H. F. (1967), *Biochemistry 6*, 1304.

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

Wasserman, R. H., and Taylor, A. N. (1968), *J. Biol. Chem. 243*, 3987.