CHARACTERIZATION OF THE METABOLITES OF VITAMIN D_3 IN THE CHICK*

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ABSTRACT

Two biologically active metabolites of vitamin D_3 have been isolated and identified as 25-hydroxy-vitamin D_3(25-OH-D_3) and 1,25-dihydroxy-vitamin D_3(1,25-diOH-D_3). 25-OH-D_3, originally denoted peak 4A, has been obtained from the blood of 1850 rachitic chicks who received a prior dose of tritiated vitamin D_3. 1,25-diOH-D_3, formerly referred to as peak 4B, was isolated from an in vitro reaction mixture containing 25-OH-D_3, a NADPH generating system and kidney homogenate from rachitic chicks. Metabolites were purified by column chromatography on silicic acid, Sephadex LH-20 and Celite, and by countercurrent distribution. 15.4μg of 25-OH-D_3 and 22.8μg of 1,25-diOH-D_3 were isolated by these techniques and characterization was carried out by ultraviolet absorption spectrophotometry and mass spectrometry. These studies confirm earlier conclusions(based upon chromatographic data)that chick metabolite 4A is identical to the 25-OH-D_3 metabolite isolated from pig blood by Blunt, DeLuca and Schnoes(Biochemistry 7, 3317(1968)). The data also substantiate the previous elucidation of the structure of chick peak 4B as 1,25-diOH-D_3 and support its role as the hormonal form of vitamin D.

INTRODUCTION

Numerous studies over the past 6 years have lead to the idea that vitamin D_3 must be metabolized to more polar sterols prior to functioning to regulate calcium transport at the intestine and skeleton. Blunt et al. (1) identified the major metabolite of vitamin D in porcine blood as 25-hydroxy-vitamin D_3(25-OH-D_3)(2)and other data suggest that this is a major circulating form in the rat(3), chick(4) and in man(5). These latter reports, however, rely on chromatographic mobility to identify 25-OH-D_3 and this metabolite has not been isolated and physically

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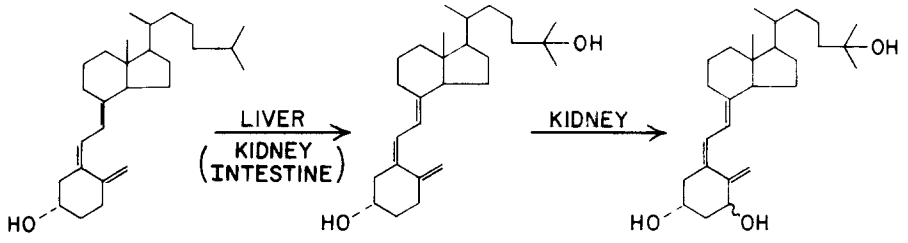
characterized from sources other than the pig. 25-OH-D_3 represents an intermediate in the conversion of vitamin D_3 to its apparent hormonal form, 1,25-dihydroxy-vitamin D_3(1,25-diOH-D_3). A current scheme for the metabolism of vitamin D_3 is shown below:



VITAMIN D_3 25-OH-D_3 1,25-diOH-D_3

The vitamin is first converted to the 25-OH-derivative in the liver, kidney or intestine(6,7); the kidney then serves as the unique endocrine organ for the production of 1,25-diOH-D_3 from 25-OH-D_3(8).

Following injection of radioactive vitamin D_3 into a vitamin D-deficient animal, 1,25-diOH-D_3 is the major form of the sterol in the target intestine(9)and is present in significant quantities in bone(10). In addition, 1,25-diOH-D_3 is the most active and fastest acting metabolite in both intestine(11) and bone(12,13). This evidence indicates that 1,25-diOH-D_3 is the sterol that mediates biochemical events which lead to calcium translocation in intestine and bone.

The present communication describes a procedure for generating extremely pure 1,25-diOH-D_3 via a renal enzyme from rachitic chicks. The first isolation and characterization of 25-OH-D_3 from chick blood is reported also.

MATERIAL AND METHODS

Materials. Animals used in all experiments were White Leghorn cockerels (kindly donated by Arizona State Hatchery) that were raised for 3-4 weeks on a vitamin D-deficient diet(14). Vitamin D_3 was purchased from Calbiochem and crystalline 25-OH-D_3 was a generous gift from Dr. John C. Babcock of the Upjohn Co. [α1,2-^{3}H] vitamin D_3(577Ci/mol) and [4-^{14}C]-vitamin D_3(32.3Ci/mol) were obtained from Amersham/Searle. [26,27-^{3}H]-25-OH-D_3(1200Ci/mol) is a product of New England Nuclear. All solvents employed for chromatographic procedures were reagent grade and were glass distilled before use.

Extraction Techniques. Methods for administration of radioactive D-vitamins, collection and extraction of blood have been described previously(10,11). In the preparation of metabolite 4A from 1850 chicks, the pooled plasma was first brought to 70% saturation with ammonium sulfate to precipitate the metabolite bound to its plasma carrier protein (1). The resulting precipitate was extracted with acetone-dichloroethane(2:1)as previously described(10)and purified according to the procedure outlined in the text.

Extraction of the aqueous kidney reaction mixture(discussed below) was carried out by adding 3.5 volumes of methanol-chloroform(2:1) to create one phase. After removal of the protein via centrifugation or filtering, 1 volume of chloroform and 1/2 volume of water were added resulting in separation of a lower, chloroform phase according to the technique of Bligh and Dyer(15). The chloroform phase contained the metabolites of interest and was dried by flash evaporation. Lipid was then solubilized with diethyl ether and the solution clarified by centrifugation at 0°C. The diethyl ether was removed by a stream of nitrogen and the samples applied to the appropriate column for purification.

Chromatographic Separations:

Silicic Acid. Silicic acid column chromatography was carried out as described by Haussler and Rasmussen(10) except that additional elution with 20% methanol in acetone and 100% methanol was included to remove the more polar plasma metabolites(peaks 4C and 4D). Silicic acid batch chromatography was performed on a 100gm silicic acid column; 100ml fractions were collected with the following elution schedule: 50% diethyl ether in hexane(fract. no. 1 to 20) and 75% diethyl ether in hexane (fract. no. 21 to 60).

Countercurrent Distribution(CCD). CCD purification of metabolite 4A was accomplished using the method of Haussler et al.(11) employing a solvent system of ethyl acetate-hexane-ethanol-water(5:15:11:9).

Sephadex LH-20. Chromatography on Sephadex LH-20 was carried out by the procedure of Holick and DeLuca(16). 1x60cm or 1.5x43cm(Fig 2A only) columns were eluted with either 50% chloroform in hexane(System I) or 65% chloroform in hexane(System II), 5ml fractions were collected with System I columns and 10ml fractions with System II columns.

Celite. Liquid-liquid partition chromatography on Celite is detailed in another publication(10). In the present experiments, two solvent schemes were utilized: System I = hexane(mobile), 15% water in methanol (stationary) and System II = 10% ethyl acetate in hexane(mobile), 45% water in ethanol(stationary).

Bioassay. Calcium absorption stimulating activity of the isolated metabolites was assessed by modification(9,11) of the procedure of Coates and Holdsworth(17).

Generation of Peak 4B, in vitro. Peak $4B_K$(18) was produced, in vitro, by a modification of the method of Fraser and Kodicek(8). The kidneys

from 12 severely rachitic chicks(30 days old; average weight=125gms; plasma $[Ca^{++}]$ =5.4mg%) were excised and homogenized with a Potter-Elvehjem homogenizer at 3°C in 10 volumes of 0.3M sucrose. The homogenate(140mls) was mixed with a phosphate buffer, pH7.4, containing Mg^{+2} to give a final volume of 540ml and final concentrations of 0.2M and 4mM for KH_2PO_4 and $MgCl_2$ respectively. NADPH generating systems were incorporated into the reaction mixture: L-malate(3.7mM), glucose-6-phosphate(1.7mM), $NADP^+$(0.15mM) and glucose-6-phosphate dehydrogenase (75 total units). The reaction was initiated by adding 150μg of [26,27-^{3}H] 25-OH-D_3(6,640dpm/μg) in 1ml of ethanol. In practice, the reaction was carried out in 5 separate flasks(250ml) with gentle shaking under air at 37°C for 2 hours. Termination of the reaction occurred with addition of methanol-chloroform(2:1) as described above.

Structure Determinations. Ultraviolet absorption studies were performed in distilled ethanol on a Cary model 15 spectrophotometer. A model RMU-6E Hitachi double focusing mass spectrometer was used to analyze approximately 5μg portions of authentic vitamin D-sterols or purified metabolites. Samples were directly introduced on the probe and continuous scanning was carried out from 80° to 150° above ambient.

RESULTS AND DISCUSSION

Isolation of Metabolite 4A. 1850 vitamin D-deficient chicks were dosed orally with 1.25μg each of(α1,2-^{3}H)vitamin D_3 (specific activity 440,000dpm/μg) and sacrificed 15-24 hrs. later. The blood harvested at decapitation was centrifuged in the presence of heparin and the resulting plasma was ammonium sulfate precipitated and processed as described in Methods. The diethyl ether solubilized, lipid extract was divided into 4 equal portions and run on 4 separate silicic acid columns(10); Fig. 1A pictures the aggregate data from these columns. Metabolite 4A is the major metabolite of vitamin D_3 in chick blood and elutes from the column with 100% diethyl ether (Fig. 1A). The metabolite from these columns was pooled and applied to a larger silicic acid column and batch eluted with 75% diethyl ether in hexane(Fig. 1B). Although at this point the metabolite was free from other vitamin D-metabolites, considerable further chromatography was required to remove other contaminating lipids. Sequential purification was carried out on CCD, Sephadex LH-20 and Celite (Figs. 1C-1F). Assuming an extinction coefficient identical to the parent vitamin D_3, 15.4μg of highly purified metabolite 4A was the estimated

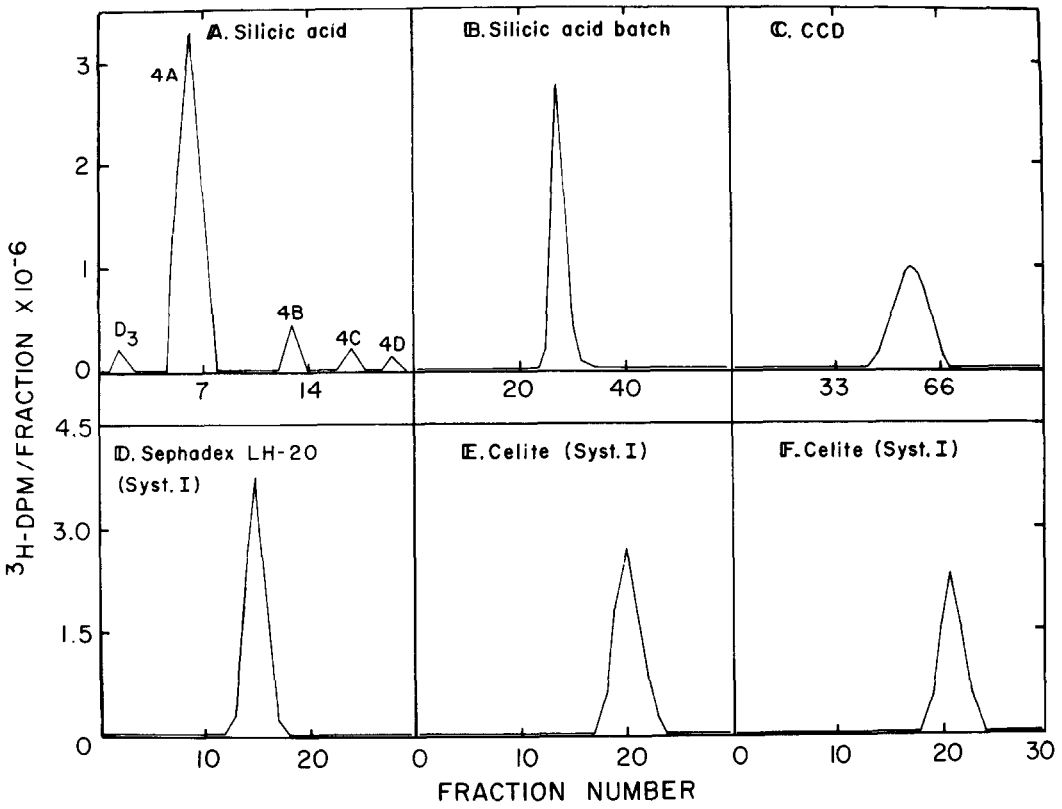


Fig. 1. Purification of metabolite 4A by chromatography. See text for details.

yield from ultraviolet absorption. Calculations of yield based upon the specific activity of the parent ^{3}H -vitamin D_3 indicated a 12% lower yield, suggesting the presence of remaining contaminants which absorb in the ultraviolet region of the spectrum.

Purification of Metabolite $4B_K$. Fig. 2 shows the results of column chromatographic isolation of metabolite $4B_K$. The metabolite was generated, in vitro, from 25-OH-D_3 as detailed in Methods. The lipid extract of the initial reaction mixture was chromatographed first on a 43cm Sephadex LH-20 column(Fig 2A); elution with 65% chloroform in hexane separated the 25-OH-D_3 substrate from its major reaction product, peak

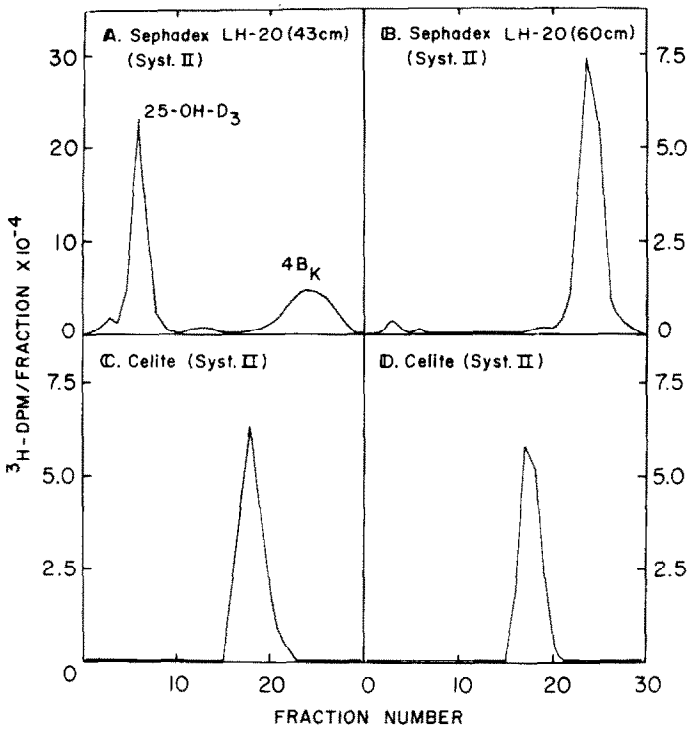


Fig. 2. Isolation of metabolite $4B_K$. See text for details.

$4B_K$. Metabolite $4B_K$ was then isolated in pure form by rechromatography on Sephadex LH-20 and elution through 2 successive Celite columns (Figs. 2B-2D). Ultraviolet absorption and calculation of yield from tritium both indicated a yield of 22.8μg of metabolite $4B_K$.

Identity of Metabolite 4B and 1,25-diOH-D_3 via Chromatography. It was of interest to insure that the metabolite of 25-OH-D_3 produced, *in vitro*, by the renal homogenate was identical to the peak 4B which was found to to be concentrated in intestinal mucosa(9). This intestinal peak 4B has been characterized recently as 1,25-diOH-D_3 by Holick *et al.*(19). Fig. 3 illustrates the results of Celite chromatography(System II) of

tritiated peak $4B_K$ along with (^{14}C)1,25-diOH-D_3 which was extracted from rachitic chick guts after treating the chicks with (^{14}C)-vitamin D_3. The (^{3}H)metabolite $4B_K$ migrates to the same chromatographic portion as

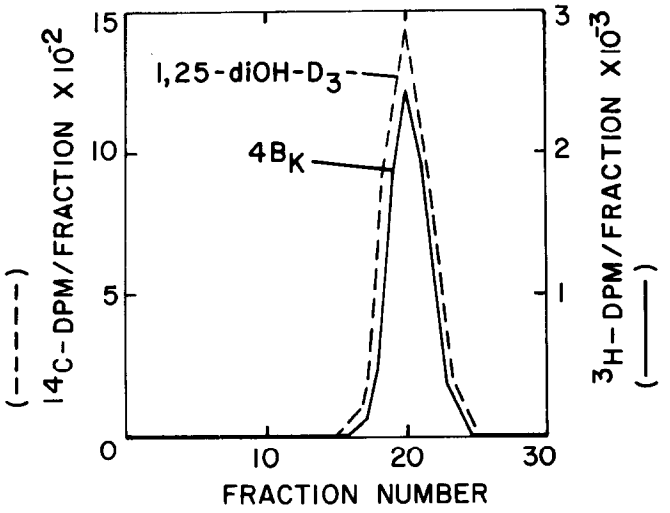


Fig. 3. Celite chromatography of (^{3}H)-metabolite $4B_K$ and (^{14}C)1,25-diOH-D_3

(^{14}C)1,25-diOH-D_3. This Celite system is presently the most powerful tool for resolving the various dihydroxy-D-vitamins(10). These data suggest that the structure of metabolite $4B_K$ is 1,25-diOH-D_3 and that this kidney metabolite is equivalent to the intestinally active form. Similar results have been reported by Norman et al.(20) and Gray et al.(21).

Biological Activity of Metabolites 4A and $4B_K$. Table I reports the results of the bioassay of metabolites 4A and $4B_K$ in terms of stimulation of intestinal transport of calcium. Metabolite 4A has very little activity 9 hrs. after administration and is fully active only after the larger dose(780 pmoles) at the 24 hr time period. Thus its time course of action closely resembles that of 25-OH-D_3(11). By contrast, metabolite $4B_K$ is more active at 9 hrs than at 24 hrs and is more effective than metabolite 4A in stimulating calcium absorption. The kinetics of action

Table I. Biological Activities of Metabolites 4A and $4B_K$

Metabolite	Sterol Dose	Time[a]	Calcium Absorption[b]
None	-	-	324 ± 60
4A	390 pmoles	9	388 ± 113
		24	754 ± 152*
	780 pmoles	9	426 ± 48+
		24	1076 ± 210*
$4B_K$	390 pmoles	9	864 ± 81*
		24	790 ± 71*
	780 pmoles	9	984 ± 148*
		24	840 ± 102*

[a]Time in hours between oral administration of metabolite and calcium transport assay.

[b]cpm ^{45}Ca/200λ plasma 40 min. after oral ^{45}Ca(11); each number is the average of 4 animals ± standard deviation.

*P<0.005 +P<0.025

of metabolite $4B_K$ are reminiscent of the rapid functioning of 1,25-diOH-D_3 (11). These data on biological activity are consistent therefore with respective structures of 25-OH-D_3 and 1,25-diOH-D_3 for metabolites 4A and $4B_K$.

Physical Characterization of Metabolites 4A and $4B_K$. Fig. 4 illustrates the ultraviolet absorption spectra of purified metabolites 4A and $4B_K$ and compares them to the spectrum of standard vitamin D_3 or 25-OH-D_3. Metabolite 4A has a spectrum close to that of vitamin D_3, with a maximum at 264nm, but the characteristic trough at 228nm is partially obscured by contaminating materials. It is tentatively concluded that the triene system is preserved in this metabolite, but mass spectrometry data is required to provide unequivocal structure elucidation. Metabolite $4B_K$

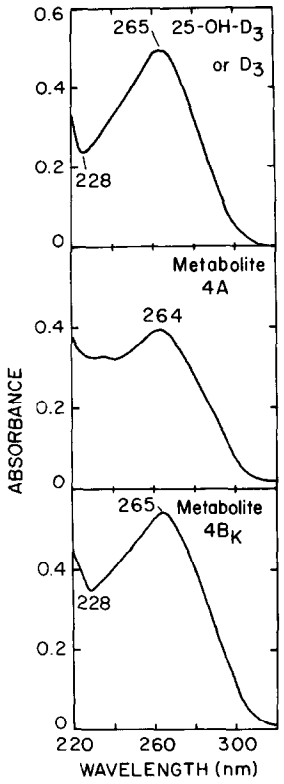


Fig. 4. UV spectra of vitamin D metabolites

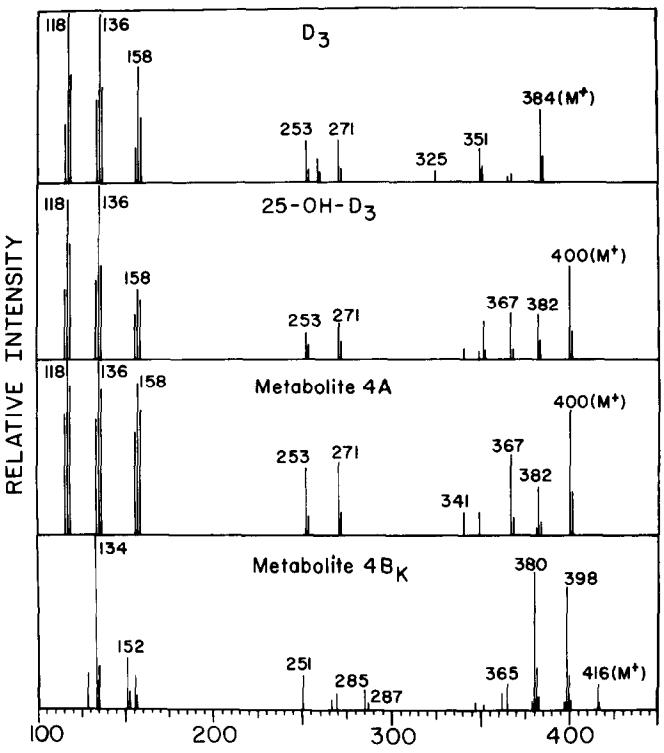


Fig. 5. Mass spectra of vitamin D_3 and its metabolites

exhibits a nearly perfect vitamin D_3 spectrum, demonstrating the retention of the 5,6-cis-triene chromophore in this metabolite.

Analysis of metabolites 4A and $4B_K$ via mass spectrometry is pictured in Fig. 5. Other workers (1,19,22) have discussed the fragmentation pattern for vitamin D_3 and its metabolites and these details will not be repeated here. Metabolite 4A displays mass fragments in a pattern identical to that of crystalline 25-OH-D_3, with characteristic ions at m/e 400 (parent), 271 and 136. The contamination which interfered with the ultraviolet absorption spectrum of metabolite 4A (Fig.4), was detected as "hydrocarbon" which vaporized from the mass spectrometer probe at

80-90°C above ambient. Followed by a period when no material was detected(90-120°C), pure metabolite 4A "puffed off" at 125°C above ambient.

Metabolite $4B_K$ emerged from the probe at 140°C above ambient and exhibited a pattern identical to that reported for 1,25-diOH-D_3(19). No unexpected or spurious fragments were seen. The fact that metabolite $4B_K$: a) yields fragments of m/e416,287, and 152 upon mass spectrometry(23) b) retains the vitamin D_3 ultraviolet absorption spectrum, c) is insensitive to clearage by periodate(10), d) has lost tritium from the number one carbon when produced from (α-1-^{3}H)vitamin D_3, and e) has biological activity characteristics analogous to 1,25-diOH-D_3, strongly supports the conclusion that this metabolite is 1,25-diOH-D_3.

Thus, the results presented here establish the structure of chick peak 4A as 25-OH-D_3 and confirm the conclusion of Lawson et al.(24) and Norman et al.(22) that chick metabolite $4B_K$ is 1,25-diOH-D_3. However, the material generated and analyzed by Norman et al.(22) was not sufficiently pure to give a discernible ultraviolet absorption spectrum. And the ultraviolet absorption spectrum reported by Lawson et al.(24) is not equivalent to that of 1,25-diOH-D_3. In addition, both of these groups were required to fractionally distill metabolite $4B_K$ from the mass probe and to correct their obtained spectra for contributing unknown contaminants. The present communication reports a generation procedure for 1,25-diOH-D_3 which results in high yields of extremely pure 1,25-diOH-D_3. By criteria of ultraviolet absorption and mass spectrometry, it is considerably purer than material previously produced in a similar fashion (22,23). Until 1,25-diOH-D_3 becomes available via chemical synthesis, metabolite obtained by this procedure will be of great use in investigating the mode of action of this hormone; it may also be of therapeutic value in patients with defective vitamin D-metabolism

and resulting bone disease.

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