Isolation and Identification of 25-Hydroxyvitamin D_2 25-Glucuronide: A Biliary Metabolite of Vitamin D_2 in the Chick†

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ABSTRACT: The biliary metabolites of vitamin D_2 obtained from chickens dosed with ^{3}H-labeled vitamin D_2 were investigated. Most of the biliary radioactivity migrated as charged compounds on diethylaminoethyl-Sephadex chromatography, and the charged fraction could be resolved into several components by reversed-phase high-pressure liquid chromatography. A major charged metabolite was further purified by reversed-phase high-pressure liquid chromatography. This compound was found to be β-glucuronidase sensitive and to yield 25-hydroxyvitamin D_2 upon mild acid hydrolysis. The metabolite was converted first to the methyl ester and then to silylated and acetylated derivatives, which were subjected to mass spectrometry. The structure of the original metabolite was established as 25-hydroxyvitamin D_2 25-β-D-glucuronic acid. This 25-hydroxyvitamin D_2 25-β-glucuronide is a major biliary metabolite of vitamin D_2 in the chick and may play a role in the chick's discrimination against vitamin D_2.

It is now widely accepted that both vitamin D_2 and vitamin D_3 must be 25-hydroxylated in the liver as the first step in their conversion to the 1,25-hydroxylated compounds believed to be the metabolites active in intestine and bone. Besides 25-hydroxyvitamin D (25-OH-D)[1] and 1,25-dihydroxyvitamin D [1,25-$(OH)_2$D], a number of other metabolites of vitamin D have been identified. [For reviews of vitamin D biochemistry, see DeLuca & Schnoes (1976) and DeLuca (1978).] Despite recent intensive investigation of vitamin D metabolism, the metabolic pathways by which vitamin D compounds are excreted remain poorly understood.

Previous studies have shown that elimination via the bile appears to be the principal mechanism for excretion of vitamin D metabolites in man (Avioli et al., 1967) and in the rat (Bell & Kodicek, 1969). Bile is also the primary route for excretion of vitamin D compounds in the chicken, particularly for vitamin D_2 metabolites (Imrie et al., 1967). The identity of the vitamin D biliary metabolites is largely unknown. Avioli et al. (1967) found that in human subjects administered [^{3}H]-vitamin D_3 only 8–9% of the biliary radioactivity was extractable with chloroform and that biliary excretion of free vitamin D_3 was negligible. Upon treatment with β-glucuronidase, an additional 40% of the biliary radioactivity became chloroform soluble, but only about 5% of the liberated radioactivity appeared to be free vitamin D_3. Bell & Kodicek (1969) observed that similar profiles were obtained upon silicic acid chromatography of bile obtained from rats dosed with either [^{3}H]vitamin D_3 or [^{14}C]vitamin D_2. All of the vitamin D_3 bile metabolites were reported to be more polar than vitamin D_3 or 25-hydroxyvitamin D_3. These workers isolated a major polar metabolite of vitamin D_3 from rat bile but were able to characterize it only as a glucuronide conjugate not identical with synthetic vitamin D_3 glucuronide.

From the above studies, it is evident that little is known about vitamin D metabolites in bile other than excretion of free vitamin D and 25-OH-D is of little importance and that most of the vitamin D metabolites in bile appear to be present in conjugated form, possibly as glucuronides. In this paper, we describe the isolation of vitamin D_2 metabolites from the bile of chickens dosed with [^{3}H]vitamin D_2 and report the identification of a major metabolite as 25-hydroxyvitamin D_2 (25-OH-D_2) 25-β-D-glucuronide.

Experimental Procedures

General Procedures. Radioactivity was determined by liquid scintillation counting with a Packard Model 3255 liquid scintillation counter equipped with automatic external standardization for determining counting efficiency. Aqueous samples were counted in Aquasol (New England Nuclear, Boston, MA) while other samples were counted in a toluene solution containing 0.2% 2,5-diphenyloxazole and 0.01% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene.

High-pressure liquid chromatography (high-pressure LC) was carried out with a Waters Model ALC/GPC 204 liquid chromatograph equipped with a Waters Model 440 absorbance detector operating at 254 nm. For reversed-phase high-pressure LC, either a 0.94 × 25 cm Partisil-10 ODS-2 semipreparative column (Whatman Inc., Clifton, NJ) or a 0.46 × 25 cm Zorbax-ODS analytical column (DuPont Co., Wilmington, DE) was used. For straight-phase high-pressure LC, a 0.46 × 25 cm Zorbax-SIL analytical column (DuPont Co.) was employed. High-pressure LC grade solvents were obtained from Fisher Chemical Co. (Chicago, IL); the solvent systems used are noted below.

Ultraviolet (UV) absorption spectra were recorded with a Beckman Model 24 recording spectrophotometer. Mass spectrometry was performed with an A.E.I. MS-9 mass spectrometer equipped with a DS-50 data acquisition system, using electron-impact ionization (70 eV) and direct-probe sample introduction at 130–160 °C above ambient temperature.

Chemicals. Vitamin D_2 was obtained from the Thompson-Hayward Chemical Co. (Kansas City, KS); its purity was ascertained by UV absorption spectroscopy. [3α-^{3}H]Vitamin D_2 of specific activity 1.9 Ci/mmol was synthesized in our laboratory (S. Yamada, L. LeVan, H. F. DeLuca, and H. K. Schnoes, unpublished experiments). This compound exhibited the characteristic vitamin D *cis*-triene UV absorption spectrum and comigrated with authentic vitamin D_2 on both straight-

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[1] Abbreviations used: high-pressure LC, high-pressure liquid chromatography; DEAE, diethylaminoethyl; BSTFA, a formulation of 1% trimethylchlorosilane in bis(trimethylsilyl)trifluoroacetamide; 25-OH-D, 25-hydroxyvitamin D; 1,25-$(OH)_2$D, 1,25-dihydroxyvitamin D; 25-OH-D_2, 25-hydroxyvitamin D_2; UV, ultraviolet.

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phase high-pressure LC (Zorbax-SIL column eluted with 1% 2-propanol in hexane) and reversed-phase high-pressure LC (Zorbax-ODS column eluted with 2% water in methanol). The tritiated vitamin D_2 was purified at the onset of this study by chromatography on silica gel eluted with hexane–ether (60:40) followed by chromatography on Lipidex 5000 (Packard Instrument Co., Downers Grove, IL) eluted with hexane–chloroform (95:5). Aliquots of the purified [^{3}H]vitamin D_2 subjected to high-pressure LC with the above systems indicated greater than 98% radiochemical purity.

All chemicals and solvents used were reagent grade. Diazomethane (CH_2N_2) in ether solution was prepared by hydrolysis of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide in a Diazald kit (Aldrich Chemical Co., Milwaukee, WI). The silylating reagent BSTFA, a formulation of bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane, was obtained from Pierce Chemical Co. (Rockford, IL). β-Glucuronidase (bovine liver type B1) was purchased from Sigma Chemical Co. (St. Louis, MO). Sephadex LH-20 and DEAE-Sephadex A-25 are products of Pharmacia Fine Chemicals (Piscataway, NJ). The DEAE-Sephadex was used in the acetate form, prepared by slurrying the gel in a large excess of 1 M ammonium acetate in methanol followed by thorough washing with methanol.

Animals. One-day-old white Leghorn cockerels were obtained from Northern Hatcheries (Beaver Dam, WI). They were maintained on a vitamin D deficient soy protein diet containing 1.2% calcium and 0.47% phosphorus for 8 weeks prior to use (Omdahl et al., 1971).

Generation of Vitamin D_2 Bile Metabolites. [^{3}H]Vitamin D_2 doses were prepared by diluting purified [^{3}H]vitamin D_2 with nonradioactive vitamin D_2 to give a specific activity of 20 mCi/mmol (114000 dpm/μg) and dissolving in 95% ethanol. A group of 20 chickens each received, by wing-vein injection, 250 μg of [^{3}H]vitamin D_2 in 50 μL of 95% ethanol. Food was withheld from the chickens 6 h prior to dosing, and they were fasted for the duration of the experiment. At 24 h after dosing, the chickens were killed; their gall bladders were removed and cut open to yield bile. The bile (39 mL) was diluted with distilled water, and aliquots were taken for radioactivity determination. The diluted bile was then lyophilized to dryness, and the resulting solids were extracted with 200 mL of methanol. The methanol extract was filtered to remove insoluble material; the filter paper and residue were thoroughly washed with several portions of methanol which were then combined with the original filtrate. This combined methanol extract was concentrated in a rotary evaporator and used for chromatography.

Chromatography of Methanol Extract. The methanol extract was applied to a 3 × 22 cm column of DEAE-Sephadex A-25 (acetate form) in methanol. Neutral compounds were eluted with methanol (200 mL), and the eluting solvent was then changed to 0.4 M ammonium acetate in methanol (400 mL) in order to elute charged compounds. Fractions (5 mL) were collected, and a 25-μL aliquot of each was used for scintillation counting (column profile, Figure 1). Fractions 20–29 containing the peak I (neutral) metabolites were pooled, as were the charged peak II metabolites (fractions 71–85).

The peak II metabolites were concentrated and applied to a 2 × 78 cm column of Sephadex LH-20 eluted with methanol. A single peak of radioactivity was observed, and the radioactive fractions 24–34 (5.0-mL fractions) were pooled for further chromatography.

High-Pressure LC of Peak II (Charged) Metabolites. Following Sephadex LH-20 chromatography, the peak II charged metabolites were concentrated and divided into four equal portions. Each portion was subjected to high-pressure LC on a Partisil ODS-2 semipreparative column eluted with methanol–water (65:35) containing 10 mM ammonium bicarbonate. The flow rate was 4 mL/min, and 4.0-mL fractions were collected; a 100-μL aliquot of each was used for scintillation counting. Several radioactive peaks (designated by the letters A–D) were present in the column profiles, an example of which is shown in Figure 2. For each run, the fractions corresponding to peak C were pooled, and the peak C regions from all four high-pressure LC runs were then combined.

Table I: Action of β-Glucuronidase on Metabolite "C"

incubation	dpm in aqueous phase	dpm in ether phase
active enzyme	2955 (42%)	4069 (58%)
boiled enzyme	3784 (72%)	1476 (28%)

The peak C fraction was next subjected to high-pressure LC on a Zorbax-ODS analytical column eluted with the same solvent at a flow rate of 2 mL/min and operated in the recycle mode. A total of six passes through the column were required to resolve the radioactive peak C from two other UV-absorbing peaks. On the final pass, the peak C fraction was collected, concentrated, and used for characterization. The UV absorption spectrum of this compound in methanol showed the characteristic vitamin D absorption band at λ_{max} = 265 nm. On the basis of the UV spectrum and radioactivity measurements, a total of 19 nmol of the peak C metabolite was obtained.

Hydrolysis of Metabolite C. The metabolite was hydrolyzed by mild acid-catalyzed hydrolysis in tetrahydrofuran, a method developed for acid-sensitive steroid glucuronides (Burstein et al., 1960; Jacobsohn & Lieberman, 1962). A 5-nmol aliquot of the peak C metabolite was treated with 200 μL of 0.01 N perchloric acid in tetrahydrofuran for 80 h at room temperature (~22 °C). After neutralization with 1% aqueous sodium bicarbonate, the reaction mixture was evaporated to dryness under nitrogen, and the residue was applied to a high-pressure LC system consisting of a Zorbax-ODS column eluted with methanol–water (90:10). With this system, unreacted metabolite eluted at 4 mL, and the reaction product which eluted at 17 mL was collected. Following evaporation to dryness, the hydrolysis product was dissolved in methanol, and its UV absorption spectrum was recorded. The product was then subjected to final purification on a Zorbax-SIL column eluted with 4% 2-propanol in hexane, and the material from this run (elution volume = 18 mL) was collected and used for mass spectrometry.

Treatment of Metabolite C with β-Glucuronidase. The metabolite (0.25 nmol) was incubated with 500 Fishman units of β-glucuronidase (bovine liver type B1) in 1.0 mL of 0.1 M sodium acetate buffer, pH 5.0, for 3 h at 37 °C. Ether (1.5 mL) was then added, and the mixture was vortexed thoroughly and then allowed to separate. Aliquots of each phase were used for radioactivity determination. A control incubation was performed in an identical fashion except the enzyme was heated in a boiling water bath for 5 min prior to incubation. The distribution of radioactivity between the organic and aqueous phases is shown in Table I.

Chemical Modifications of Metabolite C. (A) Esterification with Diazomethane. The metabolite, dissolved in 50 μL of methanol, was treated with excess diazomethane in ether for 10 min at room temperature. Excess diazomethane and solvents were removed by evaporating the reaction mixture

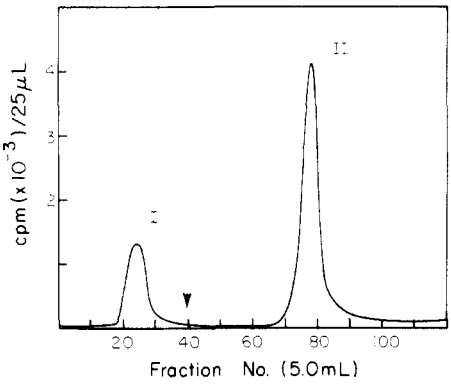

FIGURE 1: Elution profile of the methanol extract of bile on a DEAE-Sephadex column (3 × 22 cm). The arrow marks the change of eluant from methanol to 0.4 M ammonium acetate in methanol.

to dryness under a stream of nitrogen. High-pressure LC of the reaction mixture on a Zorbax-ODS column eluted with methanol–water (85:15) easily separated the less polar methylated metabolite (elution volume = 19 mL) from unreacted starting material (elution volume = 4 mL) and other impurities. In a subsequent experiment, the yield of methylated product was enhanced by including 5 μL of formic acid in the reaction mixture prior to adding diazomethane, although more of the latter must then be used. In either case, the methylated metabolite exhibited an unaltered vitamin D UV absorption spectrum with λ_{max} = 265 nm. Attempts to purify this compound further by straight-phase high-pressure LC on silica columns proved unsuccessful.

(*B*) *Trimethylsilyl Ether Formation.* The methylated metabolite (5 nmol) from (A) was treated with 50 μL of BSTFA and 50 μL of dry pyridine for 1 h at 50 °C under nitrogen. After evaporation to dryness under a stream of nitrogen, the reaction mixture was dissolved in hexane and applied to high-pressure LC. With a system consisting of a Zorbax-SIL column eluted with 0.1% 2-propanol in hexane, the silylated metabolite eluted as a single peak at 18 mL and was collected for mass spectrometry.

(*C*) *Acetylation of Metabolite.* The methylated metabolite (3 nmol) from (A) was treated with 50 μL of acetic anhydride and 50 μL of dry pyridine for 4 h at room temperature. After evaporation of the solvent to dryness under nitrogen, the product was subjected to high-pressure LC on a Zorbax-SIL column eluted with 4% 2-propanol in hexane. A single peak eluting at 17 mL was observed with the UV monitor; this material was collected and used for mass spectrometry.

Results

The bile obtained 24 h after dosing contained 6.5% of the total administered radioactivity. Chromatography of the methanol extract of bile on DEAE-Sephadex separated the radioactivity into a minor neutral fraction and a major charged fraction containing 28% and 72%, respectively, of the radioactivity recovered from the column (Figure 1). The charged fraction yielded a single peak upon Sephadex LH-20 gel filtration chromatography; recovery of radioactivity from this column was greater than 90%. Reversed-phase high-pressure LC of the peak recovered from Sephadex LH-20 resolved this charged material into several peaks (Figure 2), with the peak designated C accounting for approximately 9% of the charged metabolites. Further high-pressure LC with other reversed-phase systems demonstrated that only peaks C and D were radioactively homogeneous, while the more polar peaks A and B contained a number of radioactive metabolites. At physiological dose levels (250 ng) of [^{3}H]vitamin D_2, peak C was

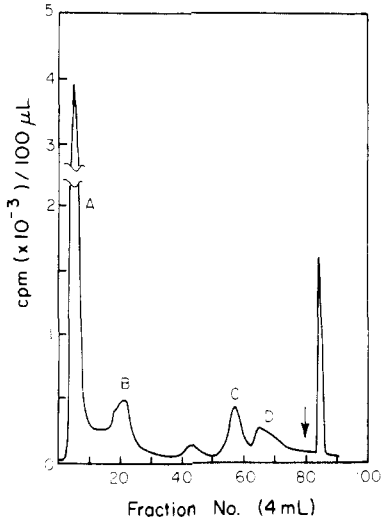

FIGURE 2: High pressure LC profile of peak II from Figure 1. A 0.94 × 25 cm ODS-2 column was eluted with methanol–water (65:35) containing 10 mM ammonium bicarbonate and then with methanol (arrow).

found to be the most abundant charged metabolite (L. LeVan and H. F. DeLuca, unpublished experiments), and for this reason we chose to characterize this compound.

Identification of Metabolite C. The purified peak C metabolite exhibited the UV absorbance spectrum (λ_{max} = 265 nm) characteristic of the vitamin D *cis*-triene system; therefore, this feature must be present in the isolated metabolite. Retention of the compound by DEAE-Sephadex indicated the presence of an acidic function. The acidic nature of the metabolite was confirmed by its reaction with diazomethane, characteristic of carboxyl groups. After methylation, the metabolite behaved as a neutral compound on DEAE-Sephadex and retained the characteristic vitamin D UV spectrum, indicating that diazomethane reacted with the carboxyl function and not with the triene system in any way.

Incubation of the metabolite with β-glucuronidase resulted in a significant increase in ether-soluble radioactivity (Table I), suggesting that the metabolite might be a glucuronide conjugate of a known vitamin D_2 compound. For this reason, the metabolite was subjected to acid-catalyzed hydrolysis under mild conditions in order to liberate the vitamin D_2 moiety for independent characterization. The chromatographic properties and UV and mass spectra of the vitamin D_2 aglycone thus obtained clearly identified this compound as 25-OH-D_2. With both high-pressure LC systems used for purification, the compound eluted at exactly the same position as synthetic 25-OH-D_2. The UV spectrum exhibited the characteristic vitamin D absorption with λ_{max} = 265 nm and λ_{min} = 228 nm while the mass spectrum (Figure 3) shows excellent agreement with the mass spectrum of 25-OH-D_2 (Suda et al., 1969).

Mass spectrometry of the intact metabolite could be performed only after esterification of the carboxyl function and silylation or acetylation of hydroxyl groups. The mass spectrum of the silylated metabolite methyl ester (Figure 4) exhibited a molecular ion at *m/e* 890, consistent with the calculated molecular weight of 890 for the tetrakis(trimethylsilyl) derivative of 25-OH-D_2 glucuronide methyl ester.

The ion at *m/e* 407 represents the tris(trimethylsilyl)glucuronic acid methyl ester fragment resulting from cleavage of the glycosidic bond at C(1) of the glucuronic acid; loss of $(CH_3)_3SiOH$ from this fragment produces the very intense peak at *m/e* 317. The latter ion is characteristic of silylated glucuronides and is usually the base peak in the mass spectra of such compounds (Billets et al., 1973). The ions at *m/e* 204

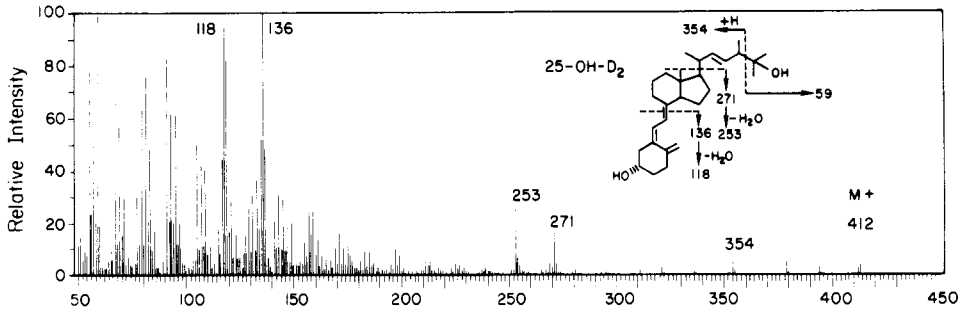

FIGURE 3: Mass spectrum of 25-OH-D_2 obtained by acid hydrolysis of metabolite C.

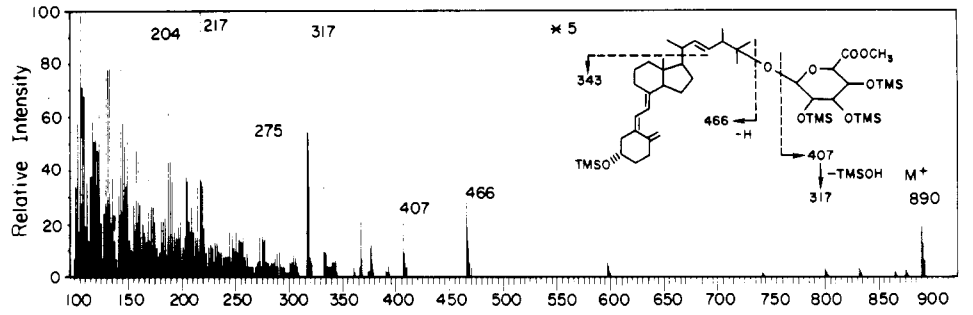

FIGURE 4: Mass spectrum and fragmentation of silylated metabolite C methyl ester. Peak heights above m/e 550 have been expanded 5-fold to facilitate examination of the spectrum.

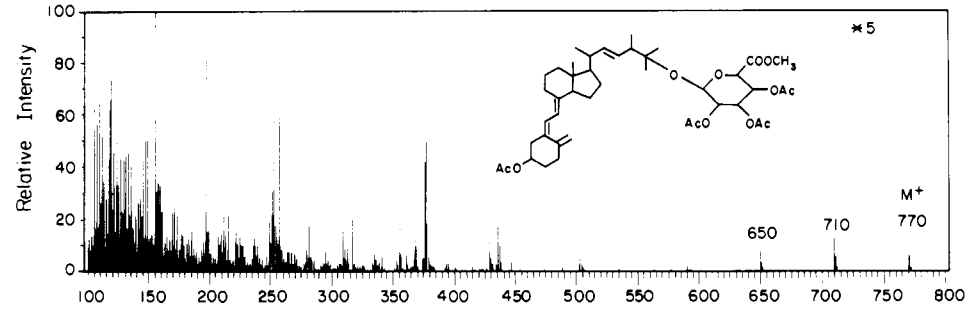

FIGURE 5: Mass spectrum and fragmentation of acetylated metabolite C methyl ester. Peak heights above m/e 750 have been expanded 5-fold to facilitate examination of the spectrum.

([$HCOSi(CH_3)_3]_2$) and m/e 217 ([$HCO(CH_3)_3Si]_2CH$) are common to all silylated carbohydrates while the ion at m/e 275 is the carboxymethyl analogue of the ion at m/e 217 (Billets et al., 1973). Elimination of the entire glucuronic acid moiety with charge retention on the vitamin D_2 fragment gives rise to the ion at m/e 466. Of interest is the ion at m/e 343, the silylated analogue of the vitamin D_2 fragment at m/e 271 (see Figure 4), which arises by loss of the side chain by cleavage of the C(17)–C(20) bond. The presence of the m/e 343 ion indicates silylation of the 3-hydroxyl group and implies the presence of a free C(3)-hydroxy group in the original metabolite. Consequently, the glucuronic acid must be conjugated to the 25-hydroxy group of the vitamin aglycon. That the side chain hydroxy function is blocked to silylation is also indicated by the absence of a peak at m/e 131 [$(CH_3)_2C = OSi(CH_3)_3^+$], which is always the base peak in the mass spectra of the trimethylsilyl derivatives of 25-hydroxycalciferols.

Additional evidence that the glucuronic acid is located at the 25 position is provided by the mass spectrum of the acetylated metabolite methyl ester (Figure 5). This compound exhibits a weak molecular ion at m/e 770 whose identity is confirmed by more prominent peaks at m/e 710 and m/e 650 resulting from the loss of one and two molecules, respectively, of acetic acid ($M_r = 60$) from the molecular ion. A molecular weight of 770 indicates addition of four acetyl groups to 25-OH-D_2 25-glucuronide methyl ester, reflecting acetylation at C-2′, C-3′, and C-4′ of glucuronic acid and at C-3 of the 25-OH-D_2 moiety. Acetylation of the alternative conjugate, 25-OH-D_2 3-glucuronide, would yield a triacetyl derivative with a molecular weight of 728 since the tertiary 25-hydroxyl is not acetylated under the mild reaction conditions employed here. Under the same conditions, authentic 25-OH-D_2 gave only the 3-monoacetylated product as determined by high-pressure LC and mass spectrometry; indeed, acetylation of the 25-hydroxyl requires much more vigorous conditions. Thus, the formation of the acetylated derivative with molecular weight of 770 supports the conclusion of a blocked 25-hydroxyl function and an available 3-hydroxyl group and, therefore, requires a 25-glucuronide structure for the metabolite. In addition, cleavage of the metabolite by β-glucuronidase implies a β-glycosidic linkage at C-1 of the glucuronic acid, since β-glucuronidase is specific for the β-glycoside configuration of glucuronic acid. Thus, the metabolite is 25-OH-D_2 25-β-glucuronide.

Discussion

In this paper, the purification of a biliary metabolite of vitamin D_2 in the chick is described. Virtually all of the vitamin D radioactivity in chick bile is methanol soluble, and it was found advantageous to resolve the components of the methanol extract into neutral and charged fractions by chromatography on DEAE-Sephadex. In this way, free (neutral) vitamin D metabolites were cleanly separated from

the conjugated (charged) metabolites, thus eliminating the ambiguous partitioning of polar compounds sometimes encountered during usual extraction procedures. Gel filtration chromatography on Sephadex LH-20 eluted with methanol was useful in removing salts and other impurities from the charged vitamin D metabolites. A rough separation of the charged metabolites was achieved by reversed-phase high-pressure LC with a semipreparative column; the peak C metabolite was then purified on an analytical high-pressure LC column. Mass spectrometry of the metabolite was successful only after conversion to its more volatile acetylated or silylated derivatives.

In the present study, the 25-OH-D_2 25-glucuronide was characterized first by hydrolysis to yield 25-OH-D_2, which was easily identified. With the identity of the vitamin D_2 moiety known, the nature and location of the conjugating group, glucuronic acid, could be ascertained from the mass spectra of the derivatized intact metabolite. In this study, the glucuronide was cleaved by use of solvent-assisted acid hydrolysis in tetrahydrofuran instead of by the more commonly used hydrolysis with β-glucuronidase. Despite the instability of vitamin D to acid conditions, the procedure described is mild enough so that no significant isomerization of the vitamin D *cis*-triene system occurred, and, thus, it may be of use for the study of other vitamin D conjugates.

In order to obtain sufficient quantities of the peak C metabolite for identification, it was necessary to dose chickens with pharmacologic doses (250 μg) of vitamin D_2. At this dose level, the peak C metabolite accounted for 9% of the charged metabolites and about 7% of the radioactivity in the bile at 24 h after dosing. Preliminary experiments demonstrated that the 25-OH-D_2 25-glucuronide is not an unnatural compound formed only at high vitamin D_2 dose levels but rather that the metabolite was present at all dose levels, its relative proportion in the bile decreasing as increasing amounts of vitamin D_2 were administered. At physiologic dose levels (250 ng of vitamin D_2), the peak C metabolite was the single most abundant vitamin D_2 metabolite in the bile, accounting for some 30% of the radioactivity recovered in the bile at 6 h after dosing. Presumably, the rate of formation of the 25-OH-D_2 25-glucuronide at high dose levels is limited by the rate of conversion of vitamin D_2 to 25-OH-D_2.

The present investigation has shown that in chickens a major route of elimination of vitamin D_2 is by conjugation of 25-OH-D_2 with glucuronic acid followed by excretion into the bile. This process is noteworthy in two respects: first, 25-OH-D_2 rather than vitamin D_2 itself is excreted; second, glucuronylation occurs at the fairly hindered 25 position.

It appears that formation of the 25-OH-D_2 25-glucuronide may play an important role in the process by which chicks and other birds discriminate against vitamin D_2. It has long been noted that vitamin D_2 is much less active than vitamin D_3 in chicks while in rats and humans the two forms of the vitamin are equally potent. Imrie et al. (1967) showed that in chicks vitamin D_2 is more rapidly removed from the blood and excreted into the bile than is vitamin D_3. Other reports (Imrie et al., 1967; Drescher et al., 1969; Jones et al., 1975, 1976a) have demonstrated that the chick is unable to raise the blood levels of vitamin D_2 metabolites to the levels observed with vitamin D_3 metabolites, while Jones et al. (1976b) showed that vitamin D_2 and vitamin D_3 are 25-hydroxylated equally well by chick liver preparations. From the present investigation, it appears that vitamin D_2 is excreted in the bile following 25-hydroxylation, in large part as the 25-glucuronide. By removal of 25-OH-D_2 from circulation, the 25-glucuronylation process reduces the blood levels of 25-OH-D_2 and also its further metabolites such as 1,25-$(OH)_2D_2$, the net result being that the target-tissue levels of the physiologically active vitamin D_2 metabolites are decreased. Preliminary experiments (L. LeVan and H. F. DeLuca, unpublished experiments) suggest that chicks dosed with physiologic amounts of [^{3}H]vitamin D_3 produce only small amounts of the 25-glucuronide, in accordance with this hypothesis. Experiments are currently in progress to compare the biliary metabolites of vitamin D_2 and vitamin D_3 in order to further examine the role of the 25-glucuronylation process in the chick's discrimination against vitamin D_2. Also of interest is whether the 25-glucuronides of vitamin D metabolites occur in mammalian bile or whether their formation is peculiar to avian species.

Acknowledgments

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