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Identification of 25,26-Dihydroxyvitamin D_3 as a Rat Renal 25-Hydroxyvitamin D_3 Metabolite†

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ABSTRACT: 25,26-Dihydroxyvitamin D_3 [25,26-$(OH)_2D_3$] was unequivocally identified as a major renal microsomal metabolite of 25-hydroxyvitamin D_3 in rats fed a vitamin D sufficient diet. The structural assignment was based on a comparison of the high-performance liquid chromatograms of synthetic and in vitro generated 25,26-$(OH)_2D_3$ through four different systems, the ultraviolet absorbance and mass spectral characteristics of biological 25,26-$(OH)_2D_3$, and the chromatographic and mass spectral characteristics of the sodium metaperiodate cleavage product of the metabolite. The enzymic synthesis of 25,26-$(OH)_2D_3$ was inhibited 60–80% by a semipurified goat anti-rat NADPH–cytochrome P-450 reductase. This implicates cytochrome P-450 as the probable terminal oxidase of the 25-hydroxyvitamin D_3-26-hydroxylase system. The methodology used to assay rat renal 25-OH-D_3-hydroxylases is also discussed.

25-Hydroxyvitamin D_3 (25-OH-D_3)[1] is the major circulating metabolite of vitamin D_3 (Haddad et al., 1977; Horst et al., 1977, 1979a). This metabolite is converted in several tissues to numerous more polar metabolites (Bikle & Rasmussen, 1974; Takasaki et al., 1978; Ribovitch & DeLuca, 1978; Norman, 1979). However, only the biological role of 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] is certain (Omdahl & DeLuca, 1973; Napoli & DeLuca, 1979; Stern, 1980). 1,25-$(OH)_2D_3$ is a hormone that mediates the traditionally measured actions of vitamin D. The function of the other metabolites, many of their structures, and, for the most part, their site(s) of synthesis are unknown. Yet evidence is accumulating to support the notion that 25-OH-D_3, and/or its metabolites other than 1,25-$(OH)_2D_3$, may be important in regulating calcium metabolism (Bordier et al., 1978; Rasmussen & Bordier, 1980). Consequently, investigation of metabolic pathways which bypass 1-hydroxylation, occurring

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[1] Abbreviations used: 25-OH-D_3, 25-hydroxyvitamin D_3; lactone, 25-hydroxyvitamin D_3 26,23-lactone; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 25,26-$(OH)_2D_3$, 25,26-dihydroxyvitamin D_3; HPLC, high-performance liquid chromatography; Mops, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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solely in mitochondria, is of interest.

Preliminary evidence had indicated the kidney microsomes convert 25-OH-D_3 to more polar, side-chain-modified compounds (Gray et al., 1978). However, none of these compounds has ever been identified. Kidney microsomes are also one locus of cytochrome(s) P-450. This family of monooxygenases catalyzes oxidations of a wide variety of substrates (Blumberg, 1978) including vitamin D and its metabolites (Napoli & DeLuca, 1979; Norman, 1979; Stern, 1980). The exact function and physiological substrates of renal microsomal cytochromes P-450, however, are not firmly established (Kupfer, 1980; Ellin & Orrenius, 1975).

This work describes a method of assaying 25-OH-D_3 metabolism in vitro using rat kidney microsomes and homogenates and demonstrates that a major rat renal microsomal product of 25-OH-D_3 metabolism is 25,26-dihydroxyvitamin D_3 [25,26-$(OH)_2D_3$]. Furthermore, evidence will be presented which suggests that the rat renal microsomal 25-hydroxyvitamin D_3-26-hydroxylase is cytochrome P-450 dependent.

Materials and Methods

General. High-performance liquid chromatography (HPLC) was done with a Waters Associates Model ALC/GPC 204 liquid chromatograph. Samples were detected with an ultraviolet absorbance detector at 254 nm. Normal-phase columns were microparticulate silica gel, Du Pont Zorbax-Sil analytical (0.46 × 25 cm), or semipreparative (0.6 × 25 cm). The reverse-phase column used was a Waters 8-mm radial compression module cartridge packed with 10-μm particles composed of octadecylsilane bonded to silica gel. All solvents were either reagent grade and distilled in glass or were HPLC grade. Radioactivity was counted with a Beckman Model LS7000 liquid scintillation counter with automatic quench control at an efficiency of 47%. Liquid scintillation fluid was prepared by dissolving 0.1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene and 2 g of 2,5-diphenyloxazole per L of toluene. Ultraviolet absorbance spectra were recorded in absolute ethanol with a Beckman Model 25 recording spectrophotometer. Mass spectra were obtained at 70 eV from the solids probe of a Finnigan Model 4021 EI/CI GC/MS coupled with an Incos Data System. So that spectra could be obtained, the probe was heated from ambient to 320 °C over a 10-min period at an ionizer temperature of 250 °C.

Compounds. Crystalline 1,25-$(OH)_2D_3$, 25,26-$(OH)_2D_3$, and 24,25-$(OH)_2D_3$ were gifts from Dr. Milan R. Uskokovic', Hoffmann-La Roche, Nutley, NJ. Crystalline 25-OH-D_3 was a gift from Dr. John Babcock, The Upjohn Co., Kalamazoo, MI. 23,25-Dihydroxy-26-carboxyvitamin D_3-26,23-lactone (lactone) was isolated from the blood of vitamin D_3 treated pigs by the procedure of Horst (1979). 25,26-$(OH)_2$[23,24-$^3H_2$]D_3 (110 Ci/mmol) was prepared from 25-OH-[23,24-$^3H_2$]D_3 (R. L. Horst, E. T. Littledike, J. L. Riley, and J. L. Napoli unpublished results). Concentrations of vitamin D_3 metabolites were determined in ethanol by UV absorbance at 265 nm with a molar extinction coefficient of 18 200.

Animals. Male rats weighing 120 ± 10 g were purchased from the Holtzman Co., Madison, WI. They were housed in groups of eight in overhanging wire cages and fed a standard rodent diet.

Anti-Reductase. Goat anti-rat γ-globulin raised against purified hepatic microsomal NADPH–cytochrome (anti-reductase) was purified by the procedure of Masters (Masters et al., 1971; Masters, 1977). For controls, an immunoglobulin G fraction was prepared from goat serum prior to inoculation with NADPH–cytochrome P-450 reductase (preimmune protein). Ten milligrams of either anti-reductase or nonimmune protein was used per mL of incubation mixture.

Homogenates. Rats (8–12/group) were killed by decapitation. Their kidneys were removed, placed on ice, decapsulated, rinsed with 0.9% sodium chloride, weighed, and minced. A glass Potter-Elvehjem homogenizer with a Teflon pestle was used to prepare a 20% (w/v) homogenate in a buffer consisting of 20 mM Mops, 250 mM sucrose, and 1 mM EDTA, pH 7.4 (buffer A). Protein was measured by the method of Lowry (Lowry et al., 1951) using human serum albumin as the standard.

Microsomes. The homogenate was spun for 15 min at $5000g_{av}$ with a JA-20 rotor in a Beckman Model J21B centrifuge. The supernatant was collected and spun for 15 min at $18000g_{av}$. The resulting supernatant was spun for 60 min at $78000g_{av}$ with a type 30 rotor in a Beckman Model L5-50 ultracentrifuge. The supernatant was decanted, and the pellet was resuspended in a buffer consisting of 100 mM Mops and 0.1 mM EDTA, pH 7.4 (buffer B). The suspension was respun for 60 min at $78000g_{av}$. The pellet was homogenized in buffer B to a final protein concentration of 8–16 mg/mL. Microsomes were essentially free of mitochondria as demonstrated by a cytochrome oxidase activity (Smith, 1955) of less than 0.05 μmol of cytochrome *c* oxidized min−1 (mg of protein).$^{-1}$ The mitochondria-containing fraction prepared at the same time (pellet from the first low-speed spin) had an activity of 3.2 μmol of cytochrome *c* oxidized min^{-1} (mg of protein)$^{-1}$.

Incubations. 25-OH-D_3 (20 μg/mg of protein) in ethanol was added to 25-mL Erlenmeyer flasks. The ethanol was evaporated, and the microsomes or homogenate was then added, followed by the remainder of the reaction materials. Final concentrations for the microsomal incubations were 90 mM Mops, pH 7.4, 0.09 mM EDTA, 10 mM magnesium acetate, 40 mM isocitrate, 2 mM NADPH, 2.4 units of isocitrate dehydrogenase, and 1 mg of microsomal protein/mL. The total incubation mixture was 2 mL/flask. For homogenate incubations, malate and NADH in final concentrations of 2 mM each were added to the incubation solution. For the boiled enzyme controls, homogenates or microsomes were immersed in a boiling water bath for 10 min. The reactions were allowed to proceed for 60 min at 37 °C with shaking in a Forma Model 2564 shaker bath. Reactions were quenched by the addition of ethyl acetate (3 mL) with vigorous mixing. [$^3H_2$]-25,26-$(OH)_2D_3$ (1000 cpm) in ethanol (25 μL) was added to the aqueous phases to quantify recovery of product, and the entire contents of the flasks were removed to screw-top test tubes and mixed well. The phases were separated and the aqueous phases were extracted twice more.

Analyses. Samples were filtered through a micro silica gel column (0.5 × 5 cm) in ethyl acetate. The ethyl acetate was evaporated, and the residue was dissolved in 100 μL of hexane–methanol–chloroform (22:1:2) and applied to a normal-phase analytical HPLC column (system 1). The 25,26-$(OH)_2D_3$ elution area was collected, the solvent was evaporated, and the residue was dissolved in 100 μL of 2-propanol–hexane (9:91) and reinjected into HPLC (system 2). In the analyses of enzyme activity, the 25,26-$(OH)_2D_3$ was collected and counted for radioactivity to quantify recovery. Total 25,26-$(OH)_2D_3$ recovered was determined from a standard curve relating nanograms of 25,26-$(OH)_2D_3$ to HPLC peak height.

Sodium Metaperiodate Reaction. Synthetic 25,26-$(OH)_2D_3$ (200 ng) or isolated 25,26-$(OH)_2D_3$ (100 ng) was dissolved in 50 μL of methanol. Fifty microliters of 5% aqueous (w/v) sodium metaperiodate were was added, and the reaction was allowed to proceed for 20 min at room temperature. The

Table I: Dependency of 25-Hydroxyvitamin D_3-26-hydroxylase Activity [pmol min^{-1} (mg of Protein)$^{-1}$] on NADPH–Cytochrome P-450 Reductase[a]

	$25,26\text{-}(OH)_2D_3$ synthesis					
expt	boiled microsomes	intact microsomes	microsomes + anti-reductase	% control act.	microsomes + nonimmune protein	% control act.
1	0.2	1.4	0.4	29	1.8	129
2	0.2	1.4	0.3	21	1.1	79

[a] The experiments were performed on two separate occasions by using microsomes prepared from at least 10 rats per preparation. Incubations were performed in duplicate or triplicate. After extraction replicates were combined.

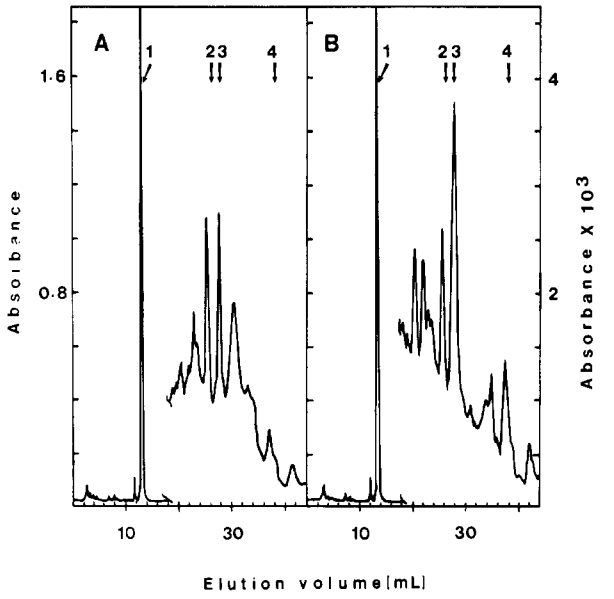

FIGURE 1: HPLC analysis (system 1) of the ethyl acetate extracts of rat kidney homogenates after incubation with 25-OH-D_3. An analytical silica gel HPLC column was eluted with hexane–methanol–chloroform (22:1:2) at 2 mL/min: (A) boiled homogenate; (B) intact homogenate. The elution positions of standard compounds were as follows: 1, 25-OH-D_3 (13 mL); 2, lactone (27 mL); 3, $24,25\text{-}(OH)_2D_3$ (28 mL); 4, $25,26\text{-}(OH)_2D_3$ (38 mL). $1,25\text{-}(OH)_2D_3$ elutes in 58 mL. The scale was changed from 2.0 to 0.005 AUFS at 18 mL.

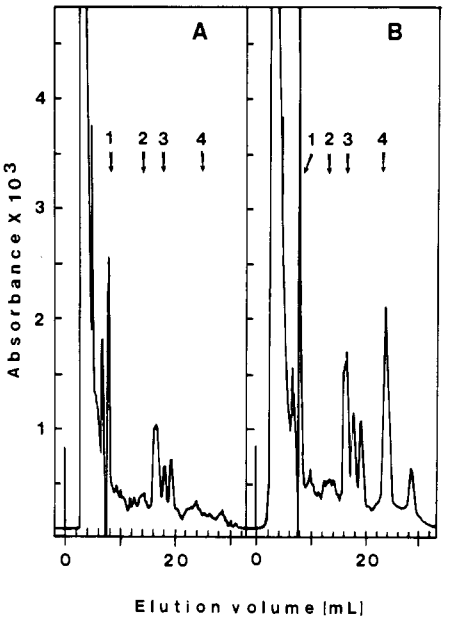

FIGURE 2: HPLC analysis (system 2) of the $25,26\text{-}(OH)_2D_3$ area (34–40 mL) recovered from column 1. An analytical silica gel HPLC column was eluted with 2-propranol–hexane (9:91) at 3 mL/min: (A) boiled homogenate; (B) intact homogenate. The elution positions of standard compounds were as follows: 1, 25-OH-D_3 (8 mL); 2, lactone (12 mL); 3, $24,25\text{-}(OH)_2D_3$ (15 mL); 4, $25,26\text{-}(OH)_2D_3$ (24 mL).

solvents were evaporated under a stream of nitrogen. The organic reaction products were separated from the inorganic salts with chloroform. The chloroform was removed, and the reaction products were dissolved in 2-propanol–hexane (2:98) and purified by HPLC.

Results

Incubation of 25-OH-D_3 with homogenates prepared from kidneys of rats maintained on a stock diet yielded several metabolites. HPLC analyses of incubation mixture extracts (system 1, Figure 1) showed that peaks at 20, 22, 28, and approximately 38 mL were enhanced in active compared to boiled homogenates. The peak at 28 mL eluted in the same volume as the $24,25\text{-}(OH)_2D_3$ standard, whereas that at 38 mL eluted in the area of standard $25,26\text{-}(OH)_2D_3$. The peaks at 20 and 22 mL did not elute with any known vitamin D compound. The other peaks, which were formed in the absence of enzyme, were substrate dependent as determined from incubations without added 25-OH-D_3.

The first HPLC system allows detection of a wide range of vitamin D metabolites, but a second system was used for precise quantification of individual peaks. In this work, the peak which coincided with $25,26\text{-}(OH)_2D_3$ was chosen for closer study. The elution area of 34–40 mL from system 1 was collected, concentrated, and reinjected onto the same analytical normal-phase column equilibrated with a different mobile phase (system 2, Figure 2). This second HPLC system again clearly demonstrated the presence of a 25-OH-D_3 rat kidney metabolite that eluted in the same volume as $25,26\text{-}(OH)_2D_3$. Moreover, the compound could be quantified from this chromatogram. For quantification, a standard curve was obtained relating peak height to nanograms of $25,26\text{-}(OH)_2D_3$ injected. It was linear from 0 to 150 ng on the 0.005 absorbance scale. It may be linear with larger amounts of $25,26\text{-}(OH)_2D_3$, but this was not checked. Recovery, determined by measuring the $[^3H_2]$-$25,26\text{-}(OH)_2D_3$ that eluted between 22 and 26 mL, was 70–90%.

This assay was used to investigate whether microsomes produced $25,26\text{-}(OH)_2D_3$ from 25-OH-D_3. The occurrence of a microsomal 25-hydroxyvitamin D_3-26-hydroxylase and its dependence on NADPH-cytochrome P-450 reductase are illustrated by the data of Table I. Clearly, an enzyme is present in rat renal microsomes that converts 25-OH-D_3 to a compound that comigrates with $25,26\text{-}(OH)_2D_3$ on two different HPLC systems. The activity of this enzyme is inhibited 70–80% by a goat anti-rat γ-globulin that was raised against purified NADPH–cytochrome P-450 reductase. The 26-hydroxylase activity was only slightly affected by an immunoglobulin G fraction prepared by the same methods from goat serum before administration of NADPH–cytochrome P-450 reductase.

For confirmation of the structure of the microsomal 25-OH-D_3 metabolite as $25,26\text{-}(OH)_2D_3$, multiple incubations

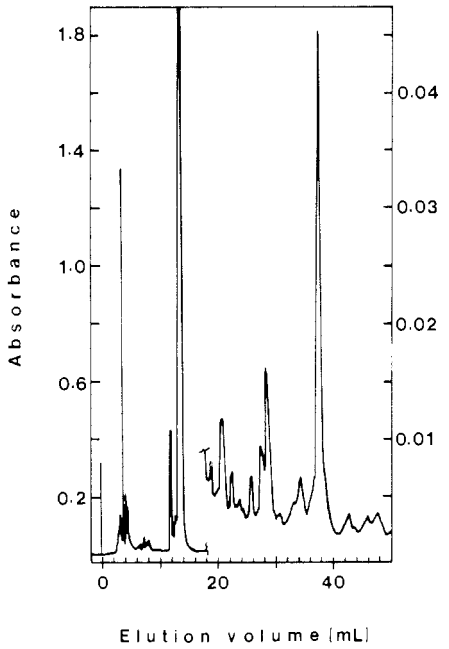

FIGURE 3: HPLC purification (system 1) of the ethyl acetate extracts of rat kidney microsomes after incubation with 25-OH-D_3. The scale was changed from 2.0 absorbance units to 0.05 absorbance units at 18 mL. The conditions are described in the legend of Figure 1.

were performed so that enough material could be obtained for isolation and spectral analyses. The combined extracts of 10 incubations were analyzed by HPLC (system 1, Figure 3). As was observed in the extracts from homogenates, most of the peaks in the microsomal extracts were substrate but not enzyme dependent. The enzyme-dependent peaks included the peak at 21 mL and the major peak at approximately 38 mL which eluted in the same volume as synthetic 25,26-$(OH)_2D_3$. The peak at 28 mL, unlike the corresponding peak detected in the homogenate extracts, was not diminished in the boiled microsome control. This suggested that the two similarly eluting peaks were hetereogeneous, and, indeed, further HPLC analysis of each demonstrated this to be the case (data not shown).

Eight more incubations were performed and the combined extracts were chromatographed like the previous batch. The 25,26-$(OH)_2D_3$ elution areas, 35–41 mL, of each batch were combined and reinjected into a system 2 that differed from the previous system 2 only in column size, i.e., a semipreparative rather than an analytical column was used (Figure 4A). Boiled microsomal control experiments showed that the large peak eluting in the same volume as synthetic 25,26-$(OH)_2D_3$ (49 mL) was the only enzyme-dependent peak. The peak eluting at approximately 14 mL was residual 25-OH-D_3 carried over from the first HPLC system. This chromatogram illustrates the facility with which 25,26-$(OH)_2D_3$ production can be quantified. In fact, the microsomal extracts were much less contaminated with ultraviolet absorbing material at this stage than were their homogenate counterparts: compare Figures 2 and 4A.

Further purification of the putative 25,26-$(OH)_2D_3$ was affected by use of two additional HPLC systems (parts B and C of Figure 4). One depended upon a reverse-phase column and was eluted with water–methanol. The other relied upon the same normal-phase column used for systems 1 and 2 but with a mobile phase composed of 2-propanol–methylene chloride. The 25-OH-D_3 metabolite eluted in the same volume as 25,26-$(OH)_2D_3$ in these last two columns and was homogeneous. Thus, the 25,26-$(OH)_2D_3$ generated by rat renal microsomes coeluted with authentic 25,26-$(OH)_2D_3$ in four distinct HPLC systems.

Spectral analyses confirmed the HPLC evidence. An absorbance spectrum of the isolated material demonstrated the typical vitamin D *cis*-triene chromophore with a maximum at 264 nm. A mass spectrum indicated that the metabolite possessed an additional hydroxyl group in the side-chain relative to 25-OH-D_3 (Figure 5). Namely, a molecular ion at m/e 416 and peaks at m/e 271 (m/e 416 minus the side chain) and m/e 253 (m/e 271 minus water) are indicative of a side-chain-dihydroxylated vitamin D_3 derivative. The peaks at m/e 398 and 383 are produced by the elimination of water from m/e 416 and elimination of a methyl group from m/e 398, respectively. The intense peak at m/e 136 results from formal cleavage between carbons 7 and 8, yielding a fragment consisting of the A ring plus carbons 6 and 7. The base peak at m/e 118 is caused by elimination of water from m/e 136. The latter two peaks are peculiar to the vitamin D triene system (Okamura et al., 1976).

For confirmation of the position of the additional hydroxyl group, the sensitivity of the metabolite to sodium metaperiodate was compared with that of synthetic 25,26-$(OH)_2D_3$. After a 20-min reaction, both synthetic and biological samples gave rise to a new compound which eluted in the same volume (26 mL) in a normal-phase HPLC system (Figure 6). The

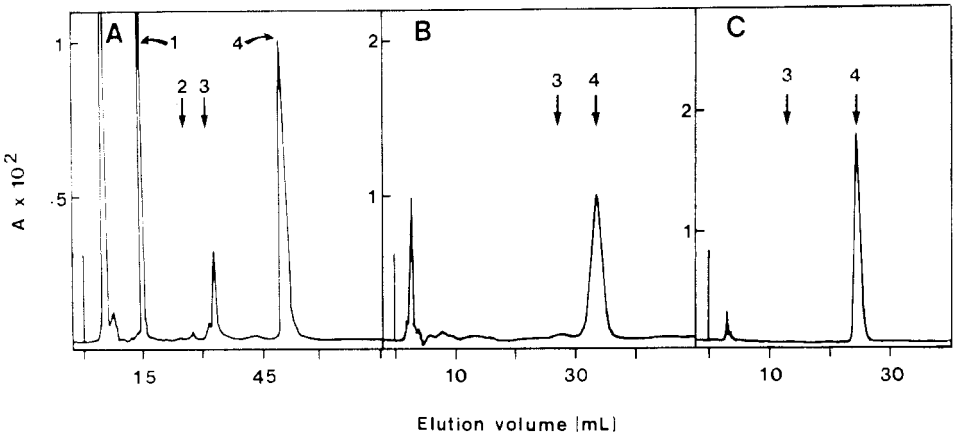

FIGURE 4: HPLC purification of materials recovered from 25,26-$(OH)_2D_3$ elution area of system 1: (A) a Du Pont semipreparative Zorbax-Sil column (0.6 × 25 cm) was eluted with 2-propranol–hexane (9:91) at a flow rate of 3.0 mL/min; (B) a Waters Associates octadecylsilane radial compression module cartridge was eluted with water–methanol (1:4) at a flow rate of 2.0 mL/min; (C) the same Du Pont analytical normal-phase column used for system 1 was eluted with 2-propanol–methylene chloride (5:95) at a flow rate of 2 mL/min. The elution positions of synthetic standards run immediately before the biological sample was loaded are marked as follows: 1, 25-OH-D_3; 2, lactone; 3, 24,25-$(OH)_2D_3$; 4, 25,26-$(OH)_2D_3$.

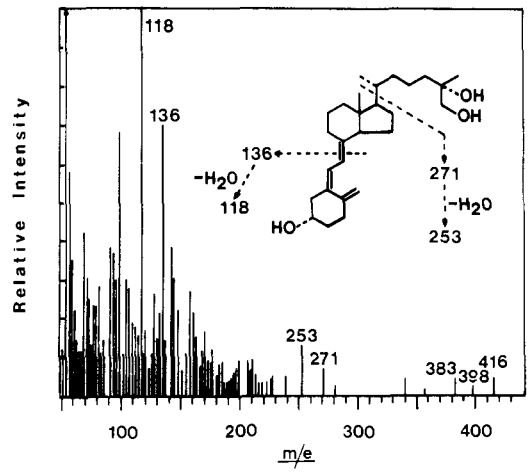

FIGURE 5: Mass spectrum of 25,26-$(OH)_2D_3$.

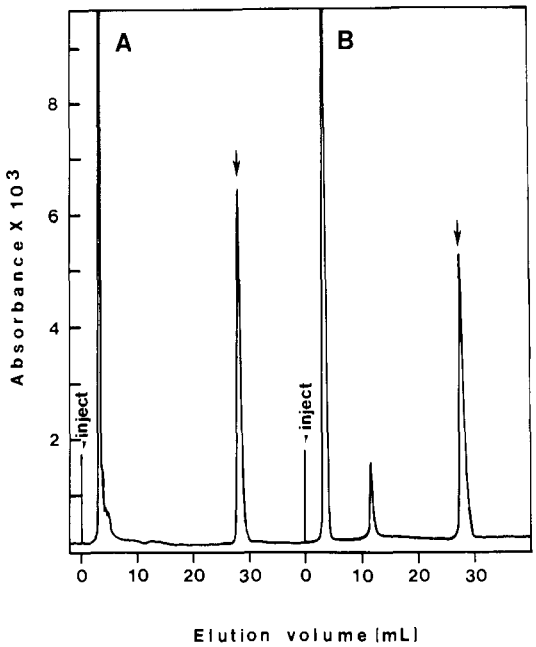

FIGURE 6: HPLC of periodate cleavage products of (A) synthetic 25,26-$(OH)_2D_3$ and (B) 25,26-$(OH)_2D_3$ generated in vitro. An analytical normal-phase column was eluted at a flow rate of 2 mL/min with 2-propanol–hexane (2:98). Arrows point to the elution positions of 27-nor-25-keto vitamin D_3.

mass spectrum of each was taken. The mass spectrum of the material generated in vitro (Figure 7) was virtually identical with that of the periodate cleavage product obtained from synthetic 25,26-$(OH)_2D_3$ (data not shown). The molecular ion at *m/e* 384 indicated that the derivative differed from the original compound by 32 amu. In other words, the original biological material was a vicinal diol and C-26 was the derivatized position. Peaks at *m/e* 366 and 351 represent losses of water and a methyl group, respectively, from the molecular ion. Loss of the side chain is indicated by *m/e* 271, whereas *m/e* 253 is caused by loss of water from *m/e* 271. The origin of the base peak at *m/e* 136 and the peak at *m/e* 118 is the same as for 25,26-$(OH)_2D_3$ itself, as discussed above.

Discussion

The evidence presented in this paper demonstrates that a major metabolite of 25-OH-D_3 produced by kidney microsomes prepared from rats on a vitamin D sufficient diet is 25,26-$(OH)_2D_3$. The structural assignment of the metabolite as 25,26-$(OH)_2D_3$ was supported by three lines of evidence.

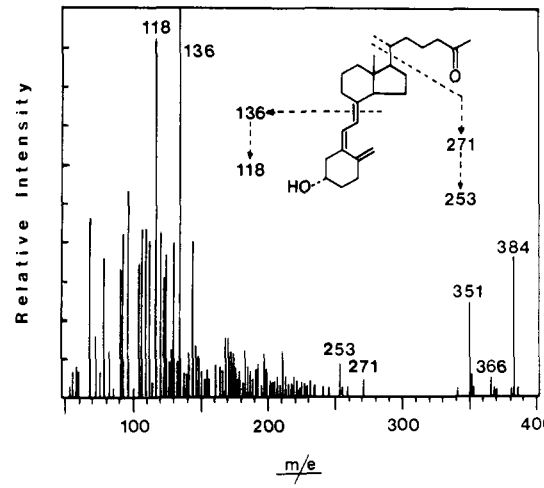

FIGURE 7: Mass spectrum of the periodate cleavage product (27-nor-25-ketovitamin D_3) obtained from 25,26-$(OH)_2D_3$ generated by rat renal microsomes.

The metabolite eluted in the same volume as synthetic 25,26-$(OH)_2D_3$ in four separate HPLC systems with different selectivities. The two normal-phase systems based on 2-propranol–hexane and 2-propanol–methylene chloride have been demonstrated repeatedly to be capable of separating the metabolites of vitamins D (Horst et al., 1981; Seamark et al., 1981). Moreover, the ultraviolet and mass spectra of the metabolite were consistent with the assigned structure. Finally, the biologically generated 25,26-$(OH)_2D_3$ yielded a periodate-cleavage product that was indistinguishable by HPLC and mass spectroscopy from that produced by synthetic 25,26-$(OH)_2D_3$. Consequently, the structural assignment as 25,26-$(OH)_2D_3$ is unambiguous. These data do not reveal whether the metabolite is 25*R* or 25*S*. However, recent work (Partridge et al., 1981) has determined that the naturally occurring configuration of 25,26-$(OH)_2D_3$ in man and in chicks is 25*S*, not 25*R* as first reported (Redel et al., 1978). This configuration is probably conserved in other species.

Little progress has been made toward understanding the possible function(s) of 25,26-$(OH)_2D_3$. Indeed, the literature contains several contradictory statements concerning this metabolite. Early reports indicated that 25,26-$(OH)_2D_3$ has intestinal-calcium transport activity but not bone-calcium mobilization activity or an ability to cure rickets (Redel et al., 1974). In fact, the compound was reported to be preferentially active on intestine (Lam et al., 1975). In contrast, more recent work has indicated that 25,26-$(OH)_2D_3$ is able to stimulate bone-calcium mobilization as well as intestinal-calcium transport if enough time is allowed for obligatory further metabolism (Thomasset et al., 1978).

23-Hydroxylation of 25,26-$(OH)_2D_3$ and oxidation of the 26-hydroxymethyl group to a carboxylic acid would yield 25-hydroxyvitamin D_3-26,23-lactone upon ring closure. Moreover, the plasma concentrations of both compounds are virtually undetectable in rats receiving small doses of vitamin D_3 but increase substantially following administration of large vitamin D_3 doses (Horst & Littledike, 1980; Shepard & DeLuka, 1980). These facts suggest that 25,26-$(OH)_2D_3$ could be an intermediate in the path of lactone synthesis. Indeed, Hollis et al. (1980) have reported that homogenates prepared from 1,25-$(OH)_2D_3$-treated chick kidneys converted 25,26-$(OH)_2D_3$ to the lactone. This experiment could not be reproduced with biologically generated, pure 25,26-$(OH)_2D_3$. Nor could the lactone be generated by dosing 25,26-$(OH)_2D_3$ to vitamin D_3 treated rats (J. L. Napoli, and R. L. Horst, unpublished observations). Consequently, 25,26-$(OH)_2D_3$ is

probably not a major intermediate in lactone synthesis but rather represents a different metabolic path.

Reports concerning the locus of the 26-hydroxylase are also inconsistent. Tanaka et al. (1978) reported preliminary evidence of a 26-hydroxylase in chick renal homogenates. But blood levels of 25,26-$(OH)_2D_3$ are not decreased in nephrectomized man (Horst et al., 1979b) or pig (Horst & Littledike, 1980). Nephrectomy decreases circulating 25,26-$(OH)_2D_3$ in rat, but this occurs concomitantly with a decrease in circulating 25-OH-D_3 (Horst & Littledike, 1980). Therefore, the connection between kidney tissue and plasma 25,26-$(OH)_2D_3$ in rat is not straightforward. Moreover, Norman (1979, p 183) has reported that the site of 25,26-$(OH)_2D_3$ is not known, but it is not a kidney metabolite. The work presented in this paper definitely establishes kidney as a site of 25,26-$(OH)_2D_3$ synthesis in rat. On the other hand, the data from nephrectomized mammals do indicate that there is also an extra renal site(s) of the 26-hydroxylase.

Cytochrome P-450 is a mixed function oxidase that catalyzes the oxidations of a wide range of steroidal hormones, many other endogenous lipids, and xenobiotics (Blumberg, 1978). Much work has been done with kidney cortex microsomal cytochromes P-450; nevertheless, their physiological substrate(s) and function(s) are still not clear (Kupfer, 1980; Anders, 1980; Ellin & Orrenius, 1975). In this work, an antibody to purified microsomal NADPH–cytochrome P-450 reductase (Yasukochi & Masters, 1976) was used to test the involvement of cytochrome P-450 in the microsomal metabolism of 25-OH-D_3. This antibody is specific to the microsomal electron transport system whose function is dependent upon the catalytic action of cytochrome P-450 (Masters; 1977; Masters et al., 1971). Consequently, inhibition of the 25-hydroxyvitamin D_3-26-hydroxylase by the anti-reductase provided forceful evidence that the hydroxylase is part of a cytochrome P-450 dependent system. Indisputable evidence will be provided only by purification and identification of the enzyme responsible or by inhibition with a specific antibody to a purified cytochrome P-450 which catalyzes this hydroxylase activity. However, if further work supports this notion, then at least one physiological role of kidney microsomal cytochrome P-450 will have been determined. Since renal microsomal cytochrome P-450, like hepatic microsomal P-450, is inducible by xenobiotics and endogenous compounds, the interesting possibility arises that 25-OH-D_3 turnover in kidney may be attenuated by a large number of organic compounds. These possibilities are now under investigation.

So that the microsomal enzymes could be studied, an assay was developed to detect and quantify rat renal 25-OH-D_3 metabolites. Traditionally, chicks have been used to study renal vitamin D-hydroxylases because of the presence of binding proteins in rat kidney that sequester substrate 25-OH-D_3 and render it unavailable for enzymic modification (Ghazarian et al., 1978). Recent efforts by several groups, including us, have overcome this problem by using high substrate concentrations or by using isolated cells (Vieth & Fraser, 1979; Turner et al., 1980; Tanaka & DeLuca, 1980). The methodology used here has the additional advantage which permits detection of a broad spectrum of 25-OH-D_3 metabolites in one HPLC pass. Discrimination by pre-HPLC columns is avoided. Therefore, it is a versatile procedure and can be used to study virtually any vitamin D hydroxylase, with only moderate modifications in the mobile phase of HPLC system 2. Moreover, this procedure does not rely on large amounts of radiolabeled vitamin D compounds. The actual expenditure of 25-OH-D_3 is also modest, since the substrate is coveniently collected during the first HPLC run. Furthermore, normal-phase HPLC is used. This has the advantages of less peak broadening, higher resolution, and cleaner chromatograms compared to reverse-phase systems. Finally, the HPLC runs are rapid.

This paper has unequivocally demonstrated that kidney is a site of 25,26-$(OH)_2D_3$ synthesis and that 25,26-$(OH)_2D_3$ is a major renal microsomal 25-OH-D_3 metabolite. Evidence for the involvement of cytochrome P-450 in the renal microsomal metabolism of 25-OH-D_3 has also been presented. Furthermore, methodology to assay rat renal 25-OH-D_3 metabolism has been developed and used to examine 25-hydroxyvitamin D_3-26-hydroxylase. Further work in this direction should aid in understanding the nature of 26-hydroxylation and its role in calcium and/or vitamin D metabolism.

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Phosphorus-31 Nuclear Magnetic Resonance Studies of the Effect of Oxygen upon Glycolysis in Yeast†

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ABSTRACT: ^{31}P NMR spectra were obtained at 145.7 MHz on suspensions of *Saccharomyces cerevisiae* cells grown on glucose, raffinose, acetate, and ethanol carbon sources. The NMR spectra were measured either under the growth conditions (i.e., using cells suspended in the growth media in the presence of the growth carbon source and O_2) or after feeding glucose under anaerobic and aerobic conditions. The NMR spectra allowed determination of intracellular pH (pH^{in}), as well as the intracellular concentrations of P_i (P_i^{in}), ATP, and other phosphorylated metabolites. In measurements performed during the steady state of glycolysis after addition of glucose, changes in P_i^{in} levels or pH^{in} were not observed upon oxygenation with glucose-repressed cells; however, in response to oxygen, derepressed cells showed a severalfold reduction in P_i^{in} concentration and an increase of pH^{in} by ~0.2–0.4 pH unit. According to in vitro data [Banuelos, M., Gancedo, C., & Gancedo, J. M. (1977) *J. Biol. Chem. 252*, 6394–6398], these particular changes of intracellular conditions should decrease the V_{max} of phosphofructokinase by a factor of ~3 and could thereby contribute appreciably to the regulation of this enzyme in the presence of oxygen. The pH^{in} values of *Saccharomyces cerevisiae* cells supplied with O_2 and the growth carbon source were found to be between 7.3 and 7.5; measurements made at extracellular pH values between 3.5 and 7.2 showed that under these conditions the pH^{in} values of these cells vary only by 0.1 pH unit. The ^{31}P NMR spectra were also measured during derepression of glucose-repressed cells; it was observed that initially intracellular pH values were ~6.7 and increased to ~7.2 slowly over a period of ~60 min. The time course of a concentrated suspension of yeast cells showed a sudden increase in pH^{in} approximately 4 min after glucose addition and time-dependent changes in P_i, fructose 1,6-bisphosphate, and ATP concentrations.

Recent experiments have demonstrated the usefulness of high-resolution ^{31}P nuclear magnetic resonance (NMR) spectroscopy for studying a variety of problems related to bioenergetics and metabolism in intact cells and tissue (Hoult et al., 1974; Salhany et al., 1975; Ugurbil et al., 1979a; Shulman et al., 1979). From the ^{31}P NMR chemical shifts of P_i and other phosphorylated metabolites with p*K*s near physiological pH values, it has been possible to determine the intracellular pH (pH^{in}). In addition, ^{31}P NMR has been used to measure the concentration of the more abundant phosphorylated metabolites (Navon et al., 1977) and to obtain information about their physical state (Ugurbil et al., 1979b) and about the rate of enzymatic reactions in vivo (Brown et al., 1977). Because all this information can be obtained simultaneously, it is possible to make time correlations between concentrations of intermediates and pH^{in}.

It is well documented that the type of carbon source used for growth by yeast cells plays an important role in determining the levels of many enzymes within these organisms [see Slonimski (1955), Perlman & Mahler (1974) and references cited therein, Mahler et al. (1975), Gancedo et al. (1965), and Rickarol & Hogan (1978)]. For example, in the presence of more than 4 mM glucose, yeast cells display enhanced levels of glycolytic enzymes and rapid rates of glycolysis while respiration is repressed; under these conditions, ethanol, the

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