C(24)- and C(23)-Oxidation, Converging Pathways of Intestinal 1,25-Dihydroxyvitamin D_3 Metabolism: Identification of 24-Keto-1,23,25-trihydroxyvitamin D_3†

Joseph L. Napoli* and Ronald L. Horst

ABSTRACT: 24-Keto-1,23,25-trihydroxyvitamin D_3 has been identified as a major 1,25-dihydroxyvitamin D_3 metabolite, produced by intestinal mucosa cells isolated from rats dosed chronically with 1,25-dihydroxyvitamin D_3. The identification was based on ultraviolet absorbance spectroscopy, mass spectroscopy, and chemical derivatization. The pathway of biosynthesis proceeded through 1,24,25-trihydroxyvitamin D_3 and 24-keto-1,25-dihydroxyvitamin D_3, which are physiological metabolites of 1,25-dihydroxyvitamin D_3. Previous work [Napoli, J. L., Pramanik, B. C., Royal, P. M., Reinhardt, T. A., & Horst, R. L. (1983) *J. Biol. Chem. 258*, 9100–9107] had shown that the amount of 24-keto-1,23,25-trihydroxyvitamin D_3 in intestine in vivo, relative to its C(24)-oxidized precursors, is enhanced by chronically dosing rats with 1,25-dihydroxyvitamin D_3. These results establish the C(24)-oxidation pathway as a predominant route of intestinal 1,25-dihydroxyvitamin D_3 metabolism under physiological conditions and indicate that treatment of the rat with exogenous 1,25-dihydroxyvitamin D_3 causes expression of C(23)-hydroxylase activity, which uses C(24)-oxidized 1,25-dihydroxyvitamin D_3 metabolites as substrates.

The compound 1,25-$(OH)_2D_3$[1] is a hormonal form of vitamin D_3 that mediates calcium and phosphate metabolism in intestine, kidney, bone, and perhaps other tissues (Kanis et al., 1982). 1,25-$(OH)_2D_3$ may also induce bone marrow cell differentiation (Miyaura et al., 1981; Abe et al., 1981). In intestine, 1,25-$(OH)_2D_3$ undergoes extensive metabolism. Some of the metabolites have been characterized, for example, 1,24,25-$(OH)_3D_3$ (Kumar et al., 1978), the C(23)-carboxylic acid, calcitroic acid (Esvelt et al., 1979), and 1,23(*S*),25-$(OH)_3D_3$ (Napoli & Horst, 1982, 1983). Many remain uncharacterized, however, and metabolic pathways have not been firmly established. Target-tissue metabolism of 1,25-$(OH)_2D_3$ may further activate the molecule and may direct it to specialized functions and is involved, most likely, in terminating its action. In any case, such metabolism would modulate the action of 1,25-$(OH)_2D_3$. Elucidation of these metabolic pathways could contribute to the understanding of the now apparent multiple functions of 1,25-$(OH)_2D_3$.

25-OH-D_3, the immediate precursor of 1,25-$(OH)_2D_3$, is converted in kidney into C(23)-, C(24)-, and C(26)-oxidized derivatives. New keto metabolites of 25-OH-D_3 have been identified, such as 23-keto-25-OH-D_3 (Horst et al., 1983), 24-keto-25-OH-D_3 (Takasaki et al., 1980, 1981), and 24-keto-23,25-$(OH)_2D_3$ (Yamada et al., 1983). The parent of the C(24)-keto compounds, 24,25-$(OH)_2D_3$, is postulated to stimulate bone mineralization (Rasmussen & Bordier, 1978; Ornoy et al., 1978), suppress parathyroid hormone secretion (Henry et al., 1977), and maintain embryonic development (Henry & Norman, 1978). The importance of metabolically modifying the C(24) alcohol to a C(24) ketone is not known, but the ketone is as active as the parent compound in the stimulation of intestinal calcium transport in rat (Takasaki, 1981). C(23)-hydroxylation, in contrast, may be a deactivation event, since 23(*S*),25-$(OH)_2D_3$ is more rapidly cleared from blood than are the other vitamin D_3 metabolites (Napoli et al., 1982a,b) and C(23)-hydroxylated derivatives are induced multifold by dosing with vitamin D metabolites (Horst & Littledike, 1980). Thus, C(24)-oxidation and C(23)-oxidation represent a branch in 25-OH-D_3 metabolism that converges in states of vitamin D excess. Analogous pathways for intestinal 1,25-$(OH)_2D_3$ metabolism might exist.

We have recently reported that 24-keto-1,25-$(OH)_2D_3$ is a rat intestinal metabolite of 1,25-$(OH)_2D_3$ under physiological circumstances (Napoli et al., 1983). During the study, a metabolite intermediate in polarity between 24-keto-1,25-$(OH)_2D_3$ and 1,24,25-$(OH)_3D_3$ was noted. The metabolite was a more abundant intestinal product in vitro, in homogenates prepared from 1,25-$(OH)_2D_3$-treated rats, than was 1,25-$(OH)_2D_3$-26,23-lactone, 1,24,25-$(OH)_3D_3$, or 24-keto-1,25-$(OH)_2D_3$. This paper will report the structure of the metabolite as 24-keto-1,23,25-$(OH)_3D_3$ and show that it is formed from 1,25-$(OH)_2D_3$ through 1,24,25-$(OH)_3D_3$ and 24-keto-1,25-$(OH)_2D_3$.

Materials and Methods

General. UV absorbance spectra were taken in 2-propanol with a Beckman Model 25 recording spectrophotometer. A molar absorptivity (ϵ) of 18 200 L mol^{-1} cm^{-1} was used for all vitamin D_3 compounds. HPLC was performed with Waters Associates ALC/GPC 204 liquid chromatographic equipment. Vitamin D compounds were detected at 254 nm. The normal-phase HPLC columns used were Du Pont Zorbax-Sil (5-μm particle; analytical, 0.45 × 25 cm; semipreparative, 0.62 × 25 cm). The analytical reverse-phase HPLC column used was a Whatman Partisil PXS 10/25 ODS-3 (0.42 × 25 cm). Solvents were distilled in glass and were filtered through a 0.45-μm filter. Silica gel Sep-Paks were purchased from Waters Associates. Radioactivity was measured in Hydro-

† From the Department of Biochemistry, The University of Texas Health Science Center, Dallas, Texas 75235 (J.L.N.), and the National Animal Disease Center, U.S. Department of Agriculture, Ames, Iowa 50010 (R.L.H.). *Received April 5, 1983.* This work was supported in part by NIH Grant AM 26535 and Robert A. Welch Foundation Grant I-797.

[1] Abbreviations: UV, ultraviolet; HPLC, high-performance liquid chromatography; DTT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; 25-OH-D_3, 25-hydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 1,24,25-$(OH)_3D_3$, 1,24(*R*),25-trihydroxyvitamin D_3; 1,23,25-$(OH)_3D_3$, 1,23(*S*),25-trihydroxyvitamin D_3; Cl-NCI, chloride ion addition, negative ion, chemical ionization; BSA, bovine serum albumin.

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Table I: Changes in Composition of Peak B with Increasing Substrate Concentration[a]

1,25-$(OH)_2D_3$ (nM)	% total metabolites in peak B[c]	% of major peak B components [pmol (30 min)$^{-1}$ (10^7 cells)$^{-1}$][b]			
		B1	B2	B3	B4
3.7	60	96 (3)	3 (0.09)	1 (0.03)	
200	52	46 (6.9)	37 (5.6)	3.5 (0.5)	13.5 (2)
1600	63	19.5 (19.6)	63 (63)	1.5 (1.5)	16 (16)

[a] 1,25-$(OH)_2D_3$ was incubated with isolated rat intestine cells for 30 min. The metabolites were separated by HPLC with a hexane-based solvent system (Figure 1). The peak B region was collected and reanalyzed with a dichloromethane-based solvent system (Figure 2). [b] From analysis of peak B region with 2-propanol/dichloromethane (Figure 2). [c] From analysis of total recovered radioactivity with an HPLC system of 2-propanol/hexane (Figure 1).

count (Baker) with a Beckman LS-330 liquid scintillation counter. Mass spectra were obtained at 70 eV from the solids probe of a Finnigan Model 4021 EI/CI GC/MS, coupled with an INCOS 2000 data system. To obtain spectra, the probe was heated from ambient temperature to 320 °C at an ionizer temperature of 250 °C. Cl-NCI mass spectra were obtained with dichlorodifluoromethane as reagent gas.

Compounds. Synthetic 1,25-$(OH)_2D_3$, 1,23(*S*),25-$(OH)_3D_3$, and 1,24(*R*),25-$(OH)_3D_3$ were gifts from Dr. Milan R. Uskokovic and Dr. John J. Partridge, Hoffmann-La Roche, Nutley, NJ. The compounds were synthesized in their laboratory by methods described previously (Partridge et al., 1976, 1981, 1982). 1,25-$(OH)_2$[26,27-^{3}H]D_3 (90 Ci/mmol) was synthesized by allowing [^{3}H]methylmagnesium bromide to react with 1α-hydroxy-27-nor-25-ketovitamin D_3. Its purity and biological activity were verified by HPLC and by its properties in 1,25-$(OH)_2D_3$ cytosolic receptor assays (Horst et al., 1981). 24-Keto-1,25-$(OH)_2$[26,27-^{3}H]D_3 and 1,24,25-$(OH)_2$[26,27-^{3}H]D_3 were prepared in vitro from 1,25-$(OH)_2$[26,27-^{3}H]D_3 (Napoli et al., 1983).

Animals. Male Sprague-Dawley rats (200–300 g), obtained from Harlan (Indianapolis, IN), were fed a stock diet. Each rat was injected intraperitoneally with 1,25-$(OH)_2D_3$ (500 ng in 0.1 mL of propylene glycol) 24, 12, and 6 h before decapitation.

Cell Preparation. The entire small intestine was removed, and the lumen was rinsed with a solution of 0.9% sodium chloride and 25 mM DTT (70 mL). The first 40 cm of the intestine was inverted and the ends were tied. Each intestinal sac was incubated with 20 mg of protease (Type IX, Sigma) for 15 min at 37 °C with shaking in 20 mL of buffer A [Hank's balanced salt solution, without calcium and magnesium, containing Hepes (15 mM), glucose (5 mM), BSA (1% w/v), penicillin G (5 IU/mL), heparin (60 USP units/mL), DTT (25 mM), and EDTA (5 mM), pH 7.4]. The mucosal cells were removed by gently pulling the intestine through the blades of a pair of scissors. The suspension was sedimented. The mucosal cells were washed with 20 mL of buffer A and were incubated with 10 mg of collagenase (from *Clostridium histdyticum*, Boehringer Mannheim) and 6 mg of hyaluronidase (Type I-S, Sigma) for 10 min at 37 °C with shaking in 20 mL of buffer B [Hank's balanced salt solution containing Hepes (15 mM), glucose (5 mM), BSA (1% w/v), penicillin G (5 IU/mL), and heparin (60 USP units/mL), pH 7.4]. The cells were filtered through three layers of coarse gauze and washed with buffer B containing PMSF (1 mM), and the cells from each intestine were suspended in 20 mL of buffer B containing 1 mM PMSF. Viability was determined by Trypan blue exclusion to be >90%.

Analysis of Metabolism. Substrate was added to an Erlenmeyer flask (125 mL) in ethanol, and the ethanol was evaporated under a stream of nitrogen. Intestinal cells were added. After incubation at 37 °C with gentle shaking, the reaction were quenched with methanol/dichloromethane (2/1), and the lipids were extracted by the procedure of Horst et al. (1981). The residue obtained from evaporation of the combined organic phases was filtered through a silica gel Sep-Pak (Waters Associates) and a 0.45-μm filter with ethyl acetate. The recovered radiolabeled material was analyzed by HPLC in the presence of unlabeled standards.

Purification of Peak B1. Cells from eight rats were prepared as described above, except the incubation with collagenase and hyaluronidase was omitted. The cells were suspended in a total of 270 mL of buffer and divided into 18 flasks, each containing 10 μg of unlabeled 1,25-$(OH)_2D_3$. After 30-min incubation, the products were extracted and filtered as described above. The recovered lipids were applied to a semipreparative HPLC column eluted with 2-propanol/hexane (1/9). The peak B metabolites eluted at 96 mL. 1,25-$(OH)_2D_3$, 24-keto-1,25-$(OH)_2D_3$, and 1,24,25-$(OH)_3D_3$ eluted at 57, 98, and 127 mL, respectively. The material that eluted between 88 and 104 mL was placed on an analytical normal-phase HPLC column developed with 2-propanol/dichloromethane (7/93). The metabolite that corresponded to peak B1 eluted at 26 mL. Several other peaks were observed. In a standardization elution, 1,25-$(OH)_2D_3$ and 1,24,25-$(OH)_3D_3$ eluted at 23 and 60 mL, respectively. Metabolite B1 was eluted through an analytical HPLC column developed with 2-propanol/hexane (3/17) in the recycle mode for a total of three passes. The purified material was used for spectral analysis.

Silylation. To 300 ng of peak B1 was added 50 μL of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide. The solution was heated at 90 °C for 135 min. The reagent was evaporated under a stream of nitrogen. The residue was applied to a reverse-phase analytical HPLC column equilibrated with 0.1% dichloromethane in methanol. The silylated metabolite eluted at 11 mL.

Results

Isolated intestinal cells from 1,25-$(OH)_2D_3$-treated rats converted 1,25-$(OH)_2$[26,27-^{3}H]D_3 into several more polar products during a 30-min incubation at a substrate concentration of 3.7 nM. Three main peaks were observed, namely, A, B, and C (Figure 1). Peak A has been identified as 24-keto-1,25-$(OH)_2D_3$; and peak C consists of 1,24,25-$(OH)_3D_3$ (94%) and 1,25-$(OH)_2D_3$-26,23-lactone (6%), when low 1,25-$(OH)_2D_3$ concentrations were incubated in a rat intestinal mucosa homogenate (Napoli et al., 1983). Peak C, the major product and the focus of this work, migrated close to 1,23,25-$(OH)_3D_3$. Upon reanalysis in a dichloromethane-based HPLC system, peak B was observed to be heterogeneous. At low substrate concentration [3.7 nM of 1,25-$(OH)_2D_3$], 96% of peak B consisted of component B1 (Table I). As the substrate concentration increased, the complexity of peak B increased. At 1600 nM, three major components, and several minor components, were noted. Peak B2 migrated close to, but was distinct from, the 1,23(*S*),25-$(OH)_3D_3$ standard

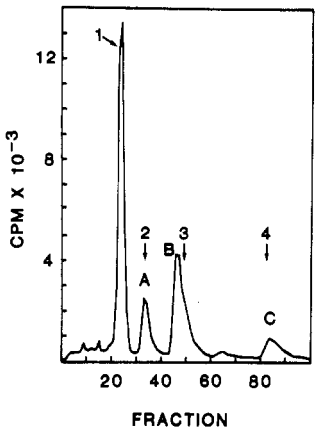

FIGURE 1: Analysis of 1,25-$(OH)_2D_3$ metabolism in isolated rat intestinal cells. 1,25-$(OH)_2$[26,27-^{3}H]D_3 (3.7 nM) was incubated for 30 min with 10^8 intestinal cells. The extract was analyzed on an analytical normal-phase HPLC column eluted with 2-propanol/hexane (1/9). Aliquots were counted for radioactivity. The elution positions of the standards are marked: (1) 1,25-$(OH)_2D_3$; (2) 24-keto-1,25-$(OH)_2D_3$; (3) 1,23,25-$(OH)_3D_3$; (4) 1,24,25-$(OH)_3D_3$. Fractions 1–18 were 2 mL each. Fractions 19–100 were 1 mL each.

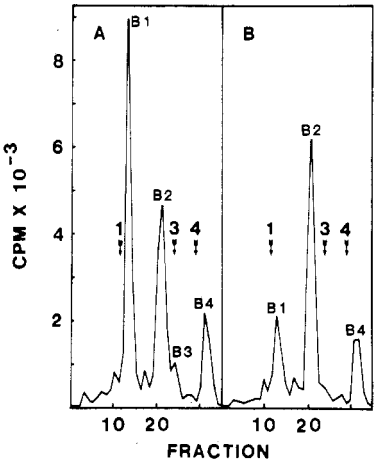

FIGURE 2: Reanalysis of peak B recovered from first HPLC analysis (Figure 1). The 1,25-$(OH)_2D_3$ concentrations in the intestinal cell incubations were 200 (panel A) and 1600 nM (panel B). Peak B was eluted from an analytical normal-phase HPLC column with 2-propanol/dichloromethane (7/93). 2-mL fractions were collected. Elution positions of standards are marked: (1) 1,25-$(OH)_2D_3$; (3) 1,23,25-$(OH)_3D_3$; (4) 1,24,25-$(OH)_3D_3$. Three major components, B1, B2, and B4, were observed, as well as several minor components, e.g., the material migrating with 1,23,25-$(OH)_2D_3$ (B3).

(Figure 2). One of the minor components, B3, however, migrated with 1,23(*S*),25-$(OH)_3D_3$ in the system shown and in two additional HPLC systems, one of which (Zorbax-NH_2) distinguished between the 23(*S*) and 23(*R*) epimers (Napoli et al., 1982a; Napoli & Horst, 1982, 1983). Component B4 did not migrate with any known 1,25-$(OH)_2D_3$ metabolite. Peak B1 appears to be the most abundant neutral intestinal 1,25-$(OH)_2D_3$ metabolite at low substrate concentrations, and therefore, it was selected for further study.

Sufficient metabolite B1 for identification was conveniently generated with the isolated intestinal cells from eight rats. The metabolite was purified with a total of three HPLC procedures. Homogeneity was ensured by cycling the metabolite through an analytical HPLC column 3 times (Figure 3). The purified metabolite had a UV absorbance spectrum with a λ_{max} at 269 nm and a λ_{min} at 230 nm. The ratio $\lambda_{max}/\lambda_{min}$ was 1.6. This is consistent with a vitamin D like 5(*E*),7,10(19)-triene chromophore. On the basis of the spectrum, approximately 1.6 μg of metabolite B1 was isolated.

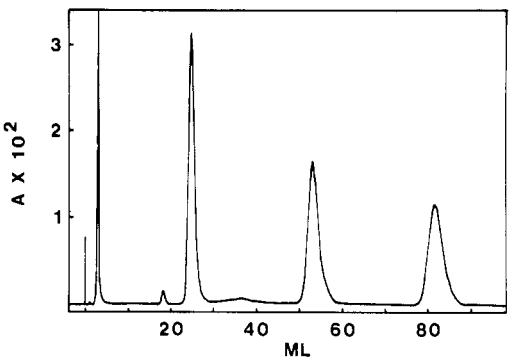

FIGURE 3: Final purification of peak B1 generated in isolated rat intestinal cells from unlabeled 1,25-$(OH)_2D_3$. An analytical normal-phase HPLC column was eluted with 2-propanol/hexane (3/17). The metabolite was recycled for a total of three passes. Authentic 1,25-$(OH)_2D_3$ eluted in 18 mL, in the first pass of a standardization elution.

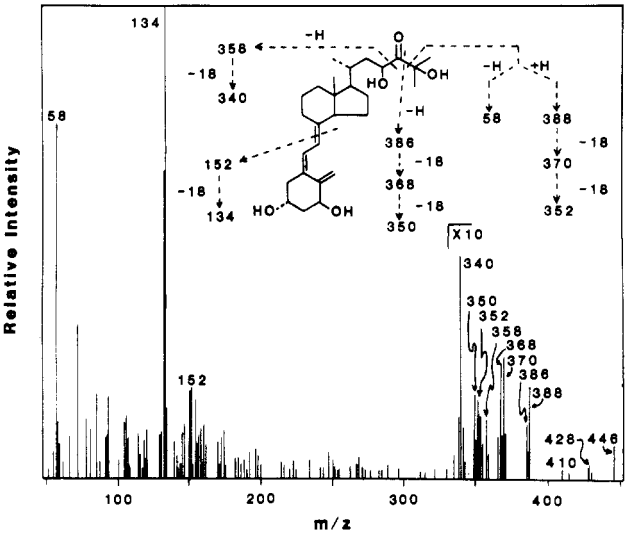

FIGURE 4: Electron-impact mass spectrum of metabolite B1.

Cl-NCI mass spectroscopy of the metabolite showed essentially four peaks. The base peak at m/z 481 (446 + ^{35}Cl) with an isotope peak at m/z 483 (446 + ^{37}Cl) indicated a molecular weight of 446. A peak at m/z 423 (60% relative intensity, 388 + ^{35}Cl) and its isotope peak at m/z 425 (338 + ^{37}Cl) resulted from loss of 58 amu from the molecular ion. This is indicative of bond cleavage between C(24) and C(25) with proton transfer from the smaller to the larger fragment, producing net loss of C_3H_6O. In other words, the C(25)-hydroxyl group is intact, and no functionality had been added to C(26) or C(27).

An electron-impact mass spectrum (Figure 4) reinforced the UV and Cl-NCI data. A molecular ion at m/z 446 was consistent with addition of two oxygen atoms, one in a keto function and the other in an alcohol group. Peaks at m/z 428 and 410 indicate sequential loss of two molecules of water from the molecular ion. A peak at m/z 152 and the base peak at 134 indicated that a 1α-hydroxylated A ring and a 5,7,10-(19)-triene were present. This suggested metabolism had occurred in the 1,25-$(OH)_2D_3$ side chain. Metabolism at C(26) or C(27) had been excluded by the Cl-NCI data. The electron-impact mass spectrum corroborated this information, since significant peaks appeared at m/e 58 and 388. Sequential loss of two molecules of water from m/z 388 provided m/z 370 and 352. Cleavage between C(24) and C(25), with proton transfer to the smaller fragment, provided m/z 386. Sequential loss of two molecules of water from m/z 386 gave m/z 368 and 350. Cleavage between C(23) and C(24) with

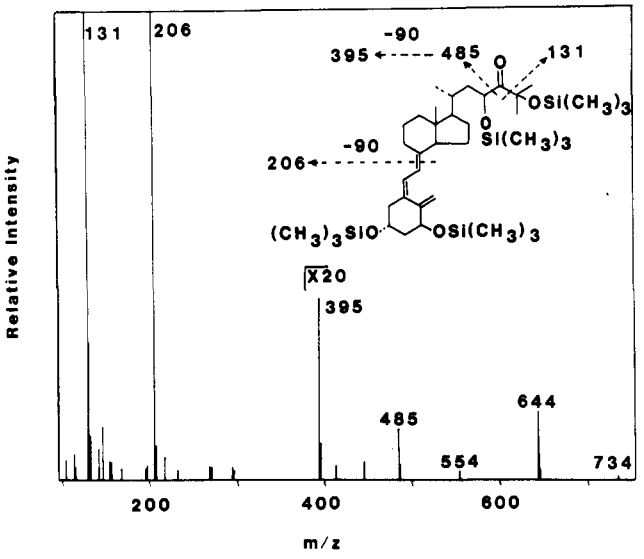

FIGURE 5: Electron-impact mass spectrum of tetrasilylated metabolite B1.

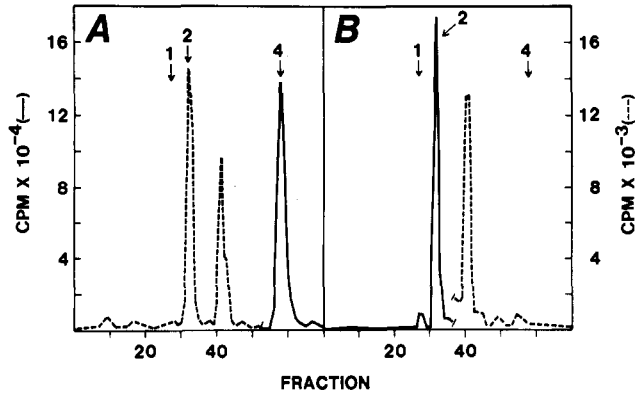

FIGURE 6: Analysis of 1,24,25-$(OH)_3$[26,27-^{3}H]D_3 (A) and 24-keto-1,25-$(OH)_2$[26,27-^{3}H]D_3 (B) metabolism by isolated rat intestinal cells. Substrates (1.3 nM) were incubated for 20 min. An analytical normal-phase column was eluted with 2-propanol/hexane (1/9), and 1-mL fractions were collected. Elution positions of standards are marked: (1) 1,25-$(OH)_2D_3$; (2) 24-keto-1,25-$(OH)_2D_3$; (4) 1,24,25-$(OH)_3D_3$. The peak eluting at 41 mL in each case is 24-keto-1,23,25-$(OH)_3D_3$.

proton transfer from the C(23) fragment yielded m/z 358. Loss of water from m/z 358 gave m/z 340. These data indicate functionalization has occurred at C(23) and C(24) and are consistent with a structure of 24-keto-1,23,25-$(OH)_3D_3$.

An electron-impact mass spectrum of the silylated metabolite had a molecular ion at m/z 734, which established that four alcohol functions were present (Figure 5). The peak at m/z 206 represented the silylated counterpart of m/z 134 in the mass spectrum of the underivatized metabolite. The peak at m/z 131 resulted from cleavage of between C(24) and C(25), i.e., loss of $C(CH_3)_2OSi(CH_3)_3$. This strengthened the conclusion that no metabolism had occurred on C(26) and C(27). The peak at m/z 644 represented loss of $(CH_3)_3SiOH$ from the molecular ion. Most significant, however, was the loss of 159 amu [$-COC(CH_3)_2OSi(CH_3)_3$] from m/z 644 to give m/z 485. This reinforces the positions of the ketone at C(24) and the second side-chain hydroxyl at C(23). Peaks at m/z 554 and 395 represent loss of $(CH_3)_3SiOH$ from m/z 644 and 485, respectively.

Conversion of 1,24,25-$(OH)_3D_3$ and 24-keto-1,25-$(OH)_2D_3$ into 24-keto-1,24,25-$(OH)_3D_3$ by rat intestinal cells would substantiate the structural assignment and would elucidate the metabolite's biosynthetic pathway. 1,24,25-$(OH)_3$[26,27-^{3}H]D_3 and 24-keto-1,25-$(OH)_2$[26,27-^{3}H]D_3 were individually incubated with isolated rat intestinal cells for 20 min. HPLC analysis of the 1,24,25-$(OH)_3$[26,27-^{3}H]D_3 incubation revealed two metabolites, corresponding to 24-keto-1,25-$(OH)_2D_3$ and 24-keto-1,23,25-$(OH)_3D_3$ (Figure 6). HPLC analysis of the 24-keto-1,25-$(OH)_2$[26,27-^{3}H]D_3 incubation revealed one metabolite, corresponding to 24-keto-1,23,24-$(OH)_3D_3$.

Discussion

This paper reports the identification of a major intestinal 1,25-$(OH)_2D_3$ metabolite as 24-keto-1,23,25-$(OH)_3D_3$. The structural assignment was based on several independent, but mutually supportive, lines of evidence. The metabolite had a UV absorbance spectrum reflective of a 5(*E*),7,10(19)-triene chromophore. The presence of the triene system was also indicated by the metabolite's mass-spectral fragmentation. The molecular weight was unequivocally established as 446 by Cl-NCI mass spectroscopy. Such a molecular weight increase, relative to 1,25-$(OH)_2D_3$, is consistent with oxidative metabolism resulting in incorporation of two oxygen atoms, one in a keto function and the other in an alcohol group. The Cl-NCI technique also indicated that the C(24)–C(25) bond of the metabolite was more labile than that of 1,25-$(OH)_2D_3$, or 1,24,25-$(OH)_3D_3$, but that no derivatization had occurred on C(26) and C(27). The electron-impact mass spectrum showed complex fragmentation, indicative of multiple side-chain functional groups, and effectively pinpointed the functionalization as C(23)-hydroxyl and C(24)-keto. These data were especially convincing considered in light of the mass spectral fragmentation patterns of 23-keto-25-OH-D_3 (Horst et al., 1983), 23-keto-1,25-$(OH)_2D_3$ (Horst et al., 1982), 24-keto-1,25-$(OH)_2D_3$ (Napoli et al., 1983), and 24-keto-23,25-$(OH)_2D_3$ (Yamada et al., 1983). The assignment was reinforced by the electron-impact mass spectrum of the silylated derivative, which showed the presence of four hydroxyl groups, and again indicated that C(26) and C(27) were not functionalized. Most importantly, the latter mass spectrum provided unequivocal evidence of C(24)-keto and C(23)-hydroxyl groups and was similar in side-chain fragmentation to the mass spectrum of silylated 24-keto-23,25-$(OH)_2D_3$ (Yamada et al., 1983). This physical evidence was corroborated by biological evidence. Both 1,24,25-$(OH)_3D_3$ and 24-keto-1,25-$(OH)_2D_3$ served as precursors to the newly identified metabolite.

Intestine is known to be a site of extensive 1,25-$(OH)_2D_3$ metabolism (Kumar et al., 1976). The existence of an intestinal 1,25-$(OH)_2D_3$-24-hydroxylase has been demonstrated (Kumar et al., 1978); but the importance of its product, 1,24,25-$(OH)_3D_3$ (Holick et al., 1973; Reinhardt et al., 1982a), to 1,25-$(OH)_2D_3$ metabolism is not clear. Recently, the intestinal generation in vitro of 1,24,25-$(OH)_3D_3$ from 1,25-$(OH)_2D_3$ at relatively high substrate concentration (1 μM) has been confirmed, and the observation has been extended to show that 1,24,25-$(OH)_3D_3$ is also produced at low (4–30 nM) substrate concentrations (Napoli et al., 1983). The same work also identified a new 1,25-$(OH)_2D_3$ intestinal metabolite as 24-keto-1,25-$(OH)_2D_3$ and showed that 1,24,25-$(OH)_3D_3$ and 24-keto-1,25-$(OH)_2D_3$ are in the pathway of intestinal 1,25-$(OH)_2D_3$ metabolism in vivo under physiological conditions. In contrast, 24-keto-1,23,25-$(OH)_3D_3$ was detected in vivo as a major 1,25-$(OH)_2D_3$ metabolite, only in intestine of rats chronically dosed with unlabeled 1,25-$(OH)_2D_3$, prior to administration of the ^{3}H-labeled 1,25-$(OH)_2D_3$.

Intestinal tissue from 1,25-$(OH)_2D_3$-treated rats produces only a modest amount of 1,23,25-$(OH)_3D_3$ (Napoli et al., 1982a; Napoli & Horst, 1982, 1983) and 1,25-$(OH)_2D_3$-26,23-lactone (Napoli et al., 1983) in vitro relative to the three C(24)-oxidized metabolites. In intestinal tissue, therefore, 1,25-$(OH)_2D_3$ metabolism seems to be directed through C-

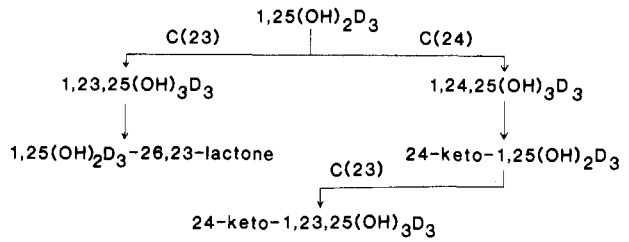

FIGURE 7: Pathways of intestinal 1,25-$(OH)_2D_3$ metabolism. Under physiological conditions, the C(24)-oxidation pathway provides 1,24,25-$(OH)_3D_3$ and 24-keto-1,25-$(OH)_2D_3$. During vitamin D_3 metabolite excess, the C(23)-oxidation is increased, resulting in accumulation of 24-keto-1,23,25-$(OH)_3D_3$ and, to a lesser extent, 1,23,25-$(OH)_3D_3$ and 1,25-$(OH)_2D_3$-26,23-lactone. Either 1,23,25-$(OH)_3D_3$ or 24-keto-1,23,25-$(OH)_3D_3$, or both, could serve as precursor to calcitroic acid.

(24)-oxidation under physiological conditions. Dosing with 1,25-$(OH)_2D_3$ apparently induced both the C(23)- and C-(24)-oxidation pathways, resulting in accumulation of C-(23)-oxidized metabolites primarily of C(24)-oxidized 1,25-$(OH)_2D_3$ derivatives and, to a much smaller extent, 1,25-$(OH)_2D_3$ itself. Therefore, intestinal 1,25-$(OH)_2D_3$ metabolism appears to follow the pathway outlined in Figure 7. Namely, during homeostasis, 1,24,25-$(OH)_2D_3$ and 24-keto-1,25-$(OH)_2D_3$ are synthesized from 1,25-$(OH)_2D_3$ by the intestine. As a consequence of vitamin D excess, the rate of C(23)-hydroxylation relative to that of C(24)-oxidation increases, and the concentrations of C(23)-hydroxylated metabolites in the intestine increase.

Other workers have concluded that a novel pathway initiated by C(23)-*ketonization* is the major intestinal route of 1,25-$(OH)_2D_3$ metabolism under physiological conditions (Ohnuma et al., 1982). In other words, C(24)-oxidation would be quantitatively minor compared to C(23)-ketonization. In contrast, we have been unable to verify the presence of detectable amounts of 23-keto-1,25-$(OH)_2D_3$ in intestine in vivo or in vitro (Napoli et al., 1983). Our search was conducted with 23-keto-1,25-$(OH)_2D_3$ standards that were synthesized in vitro from both 1,23,25-$(OH)_3D_3$ and 23-keto-25-(OH)-D_3 (Horst et al., 1982, 1983). Furthermore, our 23-keto-1,25-$(OH)_2D_3$ had a mass spectral side-chain fragmentation pattern similar to that of 23-keto-25-OH-D_3 and distinct from other 1,25-$(OH)_2D_3$ side-chain metabolites. The mass-spectral data on which Ohnuma & Norman (1982) based their structural assignments are difficult to interpret because of interfering peaks. Consequently, conclusions that C(23)-ketonization is a major pathway of intestinal 1,25-$(OH)_2D_3$ metabolism under physiological conditions should, at present, be viewed cautiously.

This work has identified a quantitatively significant intestinal metabolite of 1,25-$(OH)_2D_3$ as 24-keto-1,23,25-$(OH)_3D_3$ and has contributed to outlining C(24)-oxidation as a major pathway of intestinal 1,25-$(OH)_2D_3$ metabolism under physiological conditions. A quantitatively minor pathway involves C(23)-oxidation. The function of C(24)-oxidation is controversial, but it is interesting to note that both 1,24,25-$(OH)_3D_3$ and 24-keto-1,25-$(OH)_2D_3$ are equipotent with 1,25-$(OH)_2D_3$ in binding to its cytosolic receptor (Napoli et al., 1983). It remains possible, therefore, that C(24)-oxidation could be functional. On the other hand, 23-hydroxylated compounds are far less potent (Reinhardt et al., 1982b; Horst et al., 1983). The lower binding affinity of C(23)-oxidized 1,25-$(OH)_2D_3$ derivatives and their relatively rapid turnover rate[2] may indicate that C(23)-oxidation targets 1,25-$(OH)_2D_3$ to catabolism. The enhanced activity of the C(23)-hydroxylase after being dosed with exogenous 1,25-$(OH)_2D_3$, with resultant C(23)-oxidation of C(24)-oxidized metabolites, supports this hypothesis.

[2] R. L. Horst, and J. L. Napoli, unpublished data.

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Registry No. 24-Keto-1,23,25-$(OH)_3D_3$, 87678-01-1; 1,25-$(OH)_2D_3$, 32511-63-0; 1,24,25-$(OH)_3D_3$, 50648-94-7; 24-keto-1,25-$(OH)_2D_3$, 87678-02-2.

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Identification of Selenocysteine in Glutathione Peroxidase by Mass Spectroscopy†

Richard J. Kraus, Stephen J. Foster,‡ and Howard E. Ganther*

ABSTRACT: A convenient procedure was developed for identifying selenocysteine in selenoproteins by mass spectroscopy, based on formation of the 2,4-dinitrophenyl (DNP) derivative. Pure ovine erythrocyte glutathione peroxidase was reduced with sodium borohydride and reacted with 1-fluoro-2,4-dinitrobenzene at neutral pH under anaerobic conditions in 4 M guanidine. The inactivated enzyme was hydrolyzed with 6 N HCl for 20 h at 110 °C under anaerobic conditions. Following extraction of the hydrolysate with benzene, *Se*-(2,4-dinitrophenyl)selenocysteine in the aqueous phase was separated from non-DNP-amino acids by gel-filtration chromatography and then separated from other water-soluble DNP-amino acids by reversed-phase high-performance liquid chromatography. The *Se*-(2,4-dinitrophenyl)selenocysteine was converted to *Se*-methyl-*N*-(2,4-dinitrophenyl)selenocysteine by the addition of sodium barbital to induce an intramolecular Se → N shift (Smiles rearrangement) under anaerobic conditions, in the presence of methyl iodide to trap the liberated selenol group. Following esterification of the product's carboxyl group with methanol and hydrochloric acid, it was subjected to direct probe mass spectroscopy and identified as the methyl ester of *Se*-methyl-*N*-(2,4-dinitrophenyl)selenocysteine. This procedure allows selenocysteine to be isolated quite easily as a readily identifiable derivative and has permitted the first identification of a seleno amino acid in a protein by mass spectroscopy.

After the recognition that erythrocyte glutathione peroxidase (EC 1.11.1.9) (GSH peroxidase) was a selenium-containing enzyme (Rotruck et al., 1973; Flohe et al., 1973; Oh et al., 1974), attempts were made to identify the chemical form of the selenium moiety. Some initial attempts to identify low molecular weight forms of selenium released from the enzyme by various procedures were unsuccessful (Oh et al., 1974; Chiu et al., 1975). No seleno amino acids were detected following proteolytic digestion of GSH peroxidase that had been reduced and treated with iodoacetamide (Flohe et al., 1976). Selenocysteine was first identified in a microbial selenoprotein of the glycine reductase complex, after reduction and alkylation of the selenium to form *Se*-carboxylmethyl or *Se*-aminoethyl derivatives (Cone et al., 1976). Forstrom et al. (1978) used similar methods to derivatize the selenium in rat liver GSH peroxidase and showed that it cochromatographed with the carboxymethyl and aminoethyl derivatives of a selenocysteine standard. They were unable to obtain the mass spectrum of any selenium-containing compound from the enzyme, although they obtained mass spectra of *N*-acetyl-*O*-methyl derivatives of the Se-alkylated selenocysteine standards. Cone et al. (1976) experienced similar difficulties in their attempts to obtain mass spectra and noted the susceptibility of the selenoether derivatives of selenocysteine to oxidation. To date, cochromatography has been the primary method for identifying seleno amino acids, and no one has identified the selenium moiety of any known selenoprotein by mass spectroscopy.

We now report a new experimental approach for identification of selenocysteine in GSH peroxidase by mass spectroscopy, based on derivatization of the reduced enzyme with Sanger's reagent (FDNB)[1] to form *Se*-(2,4-dinitrophenyl)-selenocysteine. The presence of the DNP moiety made the seleno amino acid relatively easy to isolate from acid hydrolysates and permitted convenient detection during chromatography. The *Se*-DNP-selenocysteine from the enzyme was identified by mass spectroscopy following transformation to the *Se*-methyl-*N*-DNP-selenocysteine methyl ester.

Experimental Procedures

Materials. Selenocystine, FDNB, N^{ϵ}-DNP-lysine, $O^{4'}$-DNP-tyrosine, *N*-DNP-arginine, and guanidine hydrochloride (Gdn·HCl) were purchased from Sigma. N^{im}-DNP-histidine was a gift from Dr. Vincent Massey. *Se*-Methylselenocysteine was obtained from Cyclo Chemical. Nitrogen (Matheson, prepurified grade) was passed through an Oxyclear trap (Pierce Chemical). All solvents used for HPLC and mass spectroscopy were redistilled.

† From the Department of Nutritional Sciences, University of Wisconsin, Madison, Wisconsin 53706. *Received April 20, 1983.* This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, and by a grant from the National Institutes of Health (AM-14184).

‡ Postdoctoral trainee in Environmental Toxicology and Pathology.

[1] Abbreviations: FDNB, 1-fluoro-2,4-dinitrobenzene; DNP, 2,4-dinitrophenyl; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

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