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Isolation and Identification of Seven Metabolites of 25-Hydroxydihydrotachysterol$_3$ Formed in the Isolated Perfused Rat Kidney: A Model for the Study of Side-Chain Metabolism of Vitamin D†

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ABSTRACT: The in vivo metabolism of dihydrotachysterol$_3$, an analogue of vitamin D$_3$ and a potent calcemic factor, has been studied in the rat. This in vivo metabolism is compared to the in vitro metabolism of 25-hydroxydihydrotachysterol$_3$ in the perfused rat kidney. Using mass spectrometry and ultraviolet spectroscopy, we have identified seven novel metabolites derived from 25-hydroxydihydrotachysterol$_3$. The seven compounds represent intermediates on two renal pathways (24-oxidation and 26,23-lactone formation) also observed for 25-hydroxyvitamin D$_3$. No evidence was found for the renal synthesis of a 1-hydroxylated metabolite of 25-hydroxydihydrotachysterol$_3$ analogous to the hormone 1,25-dihydroxyvitamin D$_3$. Two of the compounds formed in vitro, 24,25-dihydroxydihydrotachysterol$_3$ and 25-hydroxydihydrotachysterol 26,23-lactone, were also formed in vivo. In vivo studies also revealed the formation of two other unidentified metabolites which are presumed to be formed nonrenally and may be calcemic factors. This work shows that dihydrotachysterol$_3$ metabolism is complex and probably utilizes the same side-chain enzymes as vitamin D$_3$. In addition, our work also confirms that intermediates postulated to lie on pathways to 26,23-lactone in the vitamin D$_3$ series are also formed for the side chain in dihydrotachysterol$_3$.

Although dihydrotachysterol$_2$ (DHT$_2$)[1] has been used for the treatment of hypocalcaemia associated with chronic renal disease for many decades (Cordy & Hodsman, 1984) since it was first introduced by E. Merck in 1934 (Fieser & Fieser, 1959), little is known about its metabolism or mechanism of action. Early in vivo metabolic studies were carried out with dihydrotachysterol$_3$ (DHT$_3$), which is more stable and easier to synthesize than DHT$_2$ (Hallick & DeLuca, 1971; Lawson & Bell, 1974). Both these groups reported the presence of metabolites more polar than 25-hydroxydihydrotachysterol$_3$ (25-OH-DHT$_3$), which had been shown to be formed from DHT$_3$ by rat liver homogenates (Bhattacharyya & DeLuca, 1973). Suda et al. (1970) had synthesized 25-OH-DHT$_3$ and showed that it stimulated intestinal calcium transport and bone calcium mobilization in rats. It was therefore suggested (Wing et al., 1974) that the active calcaemic metabolite of DHT was the 25-hydroxylated compound produced in the liver, which

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[1] Abbreviations: DHT, dihydrotachysterol$_2$ or dihydrotachysterol$_3$; DHT$_2$, dihydrotachysterol$_2$; DHT$_3$, dihydrotachysterol$_3$; 25-OH-DHT$_3$, 25-hydroxydihydrotachysterol$_3$; 1,25-(OH)$_2$DHT$_3$, 1,25-dihydroxydihydrotachysterol$_3$; 23,25-(OH)$_2$DHT$_3$, 23,25-dihydroxydihydrotachysterol$_3$; 24,25-(OH)$_2$DHT$_3$, 24,25-dihydroxydihydrotachysterol$_3$; 23,25,26-(OH)$_3$DHT$_3$, 23,25,26-trihydroxydihydrotachysterol$_3$; 24-oxo-25-OH-DHT$_3$, 24-oxo-25-hydroxydihydrotachysterol$_3$; 24-oxo-23,25-(OH)$_2$DHT$_3$, 24-oxo-23,25-dihydroxydihydrotachysterol$_3$; 25-OH-DHT$_3$-26,23-lactone, 25-hydroxydihydrotachysterol$_3$ 26,23-lactone; 25-OH-DHT$_3$-26,23-lactol, 25-hydroxydihydrotachysterol$_3$ 26,23-lactol; 25-OH-D$_3$, 25-hydroxyvitamin D$_3$; 1,25-(OH)$_2$D$_3$, 1,25-dihydroxyvitamin D$_3$; 23,25-(OH)$_2$D$_3$, 23,25-dihydroxyvitamin D$_3$; 24,25-(OH)$_2$D$_3$, 24,25-dihydroxyvitamin D$_3$; 25,26-(OH)$_2$D$_3$, 25,26-dihydroxyvitamin D$_3$; 24-oxo-23,25-(OH)$_2$D$_3$, 24-oxo-23,25-dihydroxyvitamin D$_3$; 25-OH-D$_3$-26,23-lactone, 25-hydroxyvitamin D$_3$ 26,23-lactone; 25-OH-D$_3$-26,23-lactol, 25-hydroxyvitamin D$_3$ 26,23-lactol; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; TSIM, (trimethylsilyl)imidazole; TMCS, trimethylchlorosilane.

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acted as a "pseudo 1α" compound because the rotation of the A-ring brings the 3-hydroxyl into the position normally occupied by the 1α-hydroxyl in 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$]. This suggestion was supported by binding studies demonstrating that 25-OH-DHT$_3$ was 10-fold more effective than 25-hydroxyvitamin D_3 (25-OH-D_3) at displacing radiolabeled 1,25-$(OH)_2D_3$ from chick duodenal receptor (Procsal et al., 1975). Such a compound would be active in anephric animals since its production requires no renal metabolism. Recent studies with DHT$_2$ have been undertaken in the rat, and 25-hydroxylated and 1,25-dihydroxylated metabolites have been putatively identified (Bosch et al., 1985a,b). This suggestion of possible 1-hydroxylation of DHT$_2$ has raised the possibility that previous theories of the mechanism of action of these vitamin D analogues are not correct and that further metabolism of 25-OH-DHT is required to produce the active calcaemic compound. If 25-OH-DHT is the active metabolite, then studies of its further catabolism are of interest. On the other hand, if 25-OH-DHT is, like 25-OH-D, merely an intermediate in the biosynthesis of an active metabolite, it is important to establish the pathway of metabolism and in what tissue it occurs. Use of structural analogues of vitamin D also provide a means of studying the specificity of the renal enzymes involved in side-chain metabolism. In an attempt to resolve these questions about the nature of the active metabolite of DHT, the in vitro metabolism of 25-OH-DHT$_3$ in the isolated perfused rat kidney has been studied, and the results have been compared with those obtained from in vivo studies using DHT$_3$.

Materials and Methods

Animals. Kidneys for perfusion studies were obtained from adult male Wistar rats (average weight 200 g) purchased from Charles River (Canada) Inc. (St-Constant, Quebec). Rats for in vivo studies were female Sprague-Dawley or Wistar rats (average weight 250 g) bred at the London Hospital Medical College. Rats were housed in animal facilities with free access to tap water and a standard rodent diet. For studies on vitamin D deprived animals, a diet deficient in vitamin D was provided for a period of 6 weeks at which time plasma concentrations of 25-OH-D were below 0.6 ng/mL.

Tachysterols. 25-Hydroxydihydrotachysterol$_3$ and [26,26,26,27,27,27-$^2H_6$]-25-hydroxydihydrotachysterol$_3$ were synthesized from intermediates obtained during the synthesis of 25-hydroxyvitamin D_3 and [26,26,26,27,27,27-$^2H_6$]-25-hydroxyvitamin D_3, respectively (Kirk et al., 1983). Irradiation of 25-hydroxy-7-dehydrocholesterol with ultraviolet light (280–320 nm) gives a number of products, among which is 25-hydroxytachysterol$_3$. After purification by high-performance liquid chromatography (HPLC), this compound was reduced with lithium/ammonia reagent as described by Hallick and DeLuca (1971) to yield 25-hydroxydihydrotachysterol$_3$ which was purified by HPLC. Hexadeuteriated 25-hydroxydihydrotachysterol$_3$ was synthesized from hexadeuteriated 25-hydroxy-7-dehydrocholesterol in a similar fashion. GC–MS of the per(trimethylsilyl) ethers of these compounds gave the expected mass spectrum with molecular ions of m/z 546 (25-OH-DHT$_3$) and 552 (hexadeuteriated 25-OH-DHT$_3$). 25-Hydroxy[26,27-^{3}H]vitamin D_3 (specific activity 130–180 Ci/mmol) was obtained from Amersham International PLC, Amersham, Bucks, U.K. Dihydrotachysterol$_3$ was a generous gift from Dr. L. Downey (Duphar Pharmaceuticals, Ltd., Southampton, U.K.).

Chemicals. Metabolites of vitamin D_3 were generous gifts from Dr. M. Uskokovic (Hoffmann-La Roche, Nutley, NJ) or were synthesized by us. Reagents used for the isolation and perfusion of rat kidneys were as previously described (Rosenthal et al., 1980). Sodium metaperiodate was purchased from BDH Chemicals Ltd. (Toronto, Ontario). Silylating reagents, *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), (trimethylsilyl)imidazole (TSIM), and BSTFA containing 1% trimethylchlorosilane (BSTFA/TMCS), and *n*-butylboronic acid were acquired from the Pierce Chemical Co. (Rockford, IL). Methoxyamine hydrochloride was obtained from Eastman Kodak Ltd. (Rochester, NY). All other reagents were of Analytical Reagent grade. Solvents were HPLC grade and were purchased from Burdick & Jackson (Muskegon, MI, or Rathburn Chemicals Ltd., Peebleshire, Scotland).

Isolated Perfused Rat Kidney. The perfused kidney system was as previously described [Rosenthal et al. (1980) for animal preparation and surgery and Reddy et al. (1982) for perfusion technique]. The rat was anaesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg body weight). A cannula was introduced into the renal artery, and perfusion was started in situ before removal of the kidney, in order to maintain a continuous oxygen supply to the tissue. The kidney was then excised and mounted in a perfusion cabinet at 37 °C. During the experiment, perfusate was recirculated through the kidney at a flow rate adjusted to maintain a corrected perfusion pressure of 80–100 mmHg. On each circulation, perfusate passed through two 1.2-μm filters and a bubble oxygenator gassed with O_2–CO_2 (19:1). The perfusion medium was a modified Krebs-Hensleit buffer adjusted to pH 7.4, containing 6% bovine serum albumin, fraction V.

25-OH-DHT$_3$. 25-OH-DHT$_3$ (50 μg) was dissolved in 100 μL of ethanol which was added to 2.0 mL of perfusion medium and introduced into the venous reservoir about 30 min after the kidney had been mounted. Hourly samples (4 mL) were taken from the venous reservoir for analysis of the time course of metabolite formation. The remaining perfusate (80–100 mL) was collected after 6 h. 25-OH-DHT$_3$ and its metabolites were extracted from perfusate medium according to the method of Bligh and Dyer (1959). the extracts were dried either by nitrogen (4-mL perfusate aliquot) or in vacuo (remaining perfusate). The residues were redissolved in hexane–2-propanol–methanol (91:7:2 v/v/v) prior to analysis and purification on HPLC.

Induction of 25-OH-D$_3$-24-hydroxylase by 25-OH-DHT$_3$. The 24-oxidation enzymes of vitamin D_3 were induced in the isolated perfused kidneys of vitamin D depleted rats by the addition of 25-OH-DHT$_3$ or other analogues of 1,25-$(OH)_2D_3$ to the perfusate 30 min after the kidneys had been mounted. In order to assay 25-OH-D_3-24-hydroxylase, we added to perfusate in 100 μL of ethanol simultaneously 1 μCi of [26,27-^{3}H]-25-OH-D_3 (20 Ci/mmol; Amersham, Arlington Heights, IL) and collected perfusate samples (4 mL) at 2, 4, and 6 h. These aliquots were subjected to lipid extraction, HPLC on Zorbax-CN, and fraction collection (40 × 0.5 min fractions) as described below. Fractions were allowed to dry in air and then subjected to liquid scintillation counting in an organic-based scintillant. ^{3}H peaks corresponding to [^{3}H]-25-OH-D_3, [^{3}H]-24,25-$(OH)_2D_3$, and [^{3}H]-1,25-$(OH)_2D_3$ were integrated and expressed as a percent total recovered radioactivity.

High-Performance Liquid Chromatography (HPLC). Analytical HPLC was carried out on systems consisting of combinations of a WISP Model 710 or 712 sample injector, a Model 510 or 590 pump, and a Model 440 fixed-wavelength detector controlled by a Model 840 chromatography data station or a Model 990 photodiode array assembly (all supplied by Waters Scientific, Milford, MA). In some separations a

Model 750/03 pump (Applied Chromatography Systems, Luton, Beds., U.K.) and a variable-wavelength detector (Model SF770 or SF789, Schoeffel Instrument Corp., Westwood, NJ) were used. Separations were obtained on a Zorbax-SIL (0.62 × 25 cm) column eluted with the solvent system hexane–2-propanol–methanol (91:7:2 v/v/v) at a flow rate of 2 mL/min or a Zorbax-CN (or occasionally Hypersil-CN) column (0.46 × 25 cm) eluted with the solvent hexane–2-propanol–methanol (94:5:1 v/v/v) at a flow rate of 1.2 mL/min (Jones, 1980). Occasionally preparative Zorbax-SIL columns (0.94 × 25 cm) were used, elution being with the 91:7:2 solvent system at a flow rate of 4 mL/min. All separations were carried out at room temperature. At an early stage of investigation 1-min fractions were collected on a Superac 2211 fraction collector (LKB, Bromma, Sweden) and appropriate peaks pooled for further study. At later stages of purification, peaks were collected in toto manually.

Diode Array Spectrophotometry. Diode array spectrophotometry was used to identify peaks as metabolites of DHT and formed the main criterion for judging the purity of peaks prior to mass spectrometry. Inspection of three-dimensional plots revealed the presence or absence of contaminating peaks with inappropriate spectra. Such scanning diode array spectrophotometry was carried out with a Model HP8450 scanning spectrophotometer connected to an HP9816 microcomputer (Hewlett-Packard, Palo Alto, CA) (Jones et al., 1986) or with the Waters Model 990 photodiode array assembly.

Periodate Oxidation. Metabolites (approximately 200 ng) were dissolved in methanol (40 μL), and sodium metaperiodate (5% w/v in water, 20 μL) was added in reduced light. The reaction mixture was vortexed and incubated in the dark for 30 min either at room temperature or at 55 °C. After the incubation, the solvent was evaporated to dryness with nitrogen. The residue was then redissolved in appropriate HPLC solvent for analysis by HPLC. For direct probe mass spectrometry, it was necessary to oxidize larger quantities of metabolites (approximately 800 ng).

Formation of Derivatives Prior to Mass Spectrometry. For direct probe mass spectrometry, samples of the purified metabolites (approximately 800 ng) were converted into their trimethylsilyl ethers by dissolving in pyridine (15 μL), adding BSTFA/TMCS reagent (10 μL), and incubating for 45 min at 55 °C. At the end of this time the solvents were evaporated in a stream of nitrogen. The residue was dissolved in hexane and was transferred to a clean dry capillary tube ready for mass spectrometry. For gas chromatography–mass spectrometry (GC–MS), samples were converted to trimethylsilyl ether derivatives by incubation with TSIM (50 μL) for 40 min at 45 °C as described by Street et al. (1986). These two procedures convert all hydroxyl groups on the steroid molecule to trimethylsilyl ethers. Incubation with BSTFA alone only forms derivatives on nonsterically hindered hydroxyl groups. Cyclic *n*-butylboronate ester–trimethylsilyl ether derivatives were obtained by reaction with *n*-butylboronic acid followed by BSTFA as described by Coldwell et al. (1984). *O*-Methyl oximes were formed on oxo groups by incubation of the sample (approximately 200 ng) with 100 μL of methoxyamine hydrochloride in pyridine (2% w/v) for 1 h at 60 °C, after which time the pyridine was removed under a stream of nitrogen and the oximes were extracted with hexane. Trimethylsilyl ethers were subsequently formed by incubation with TSIM.

Mass Spectrometry. Direct probe mass spectrometry was carried out on a Model 5985 spectrometer (Hewlett-Packard, Palo Alto, CA). Samples (400–800 ng) were dissolved in hexane or methanol and transferred to clean dry capillary tubes. These tubes were then introduced into the mass spectrometer on a direct insertion probe which was heated from a starting temperature of between 25 and 40 °C at a programmed rate of 15 °C/min. Ionization voltage was 70 eV. Positive ion EI spectra were recorded and stored on disc with a Hewlett-Packard Series 21MX-E computer. Gas chromatography–mass spectrometry was carried out on an R10-10C quadrapole spectrometer (Nermag SN, Reuil-Malmaison, France). Gas chromatography was carried out on a capillary column [CP SIL 5, Chrompak (U.K.) Ltd., London, i.d. 0.22 mm × 25 m] with a carrier gas (helium) flow rate of 1 mL/min. Samples were injected directly onto the column at a temperature of 60 °C, and the temperature was rapidly raised (40 °C/min) to 300 °C. The 24-eV positive EI spectra were obtained at the apex of each peak, and background subtraction was carried out.

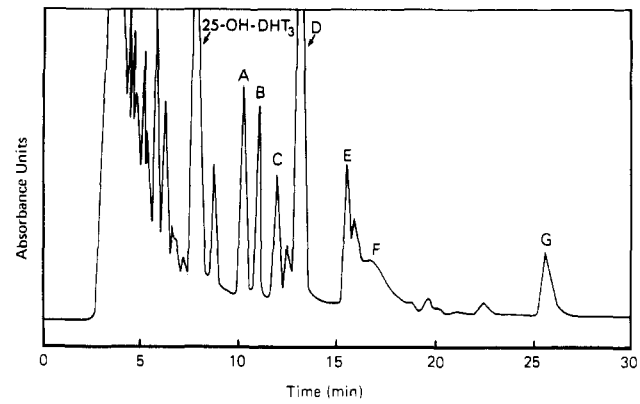

FIGURE 1: HPLC of the lipid extract of perfusate from a rat kidney perfused in the presence of 1.25 μM 25-OH-DHT_3. Conditions used were as follows: Zorbax-SIL (0.94 × 25 cm); hexane–2-propanol–methanol (91:7:2 v/v/v); 4.0 mL/min; 254 nm. Metabolites of 25-OH-DHT_3 are labeled A–G, and their subsequent identification is described in the text.

In Vivo Experiments. Vitamin D replete rats were given a single intraperitoneal injection of DHT_3 [1 mg dissolved in 500 μL of 6% (v/v) ethanol in propylene glycol]. At 18 h later, the rats were exsanguinated after ether anaesthesia. Plasma was extracted with Bond-Elut C18 cartridges as described by Coldwell et al. (1985). Metabolites were separated and purified by HPLC on Zorbax-SIL and -CN columns as described above.

Binding Studies. The affinity of rat vitamin D binding globulin (DBP) for certain tachysterol metabolites was measured by the displacement of [^{3}H]-25-OH-D_3 from 1:15 000 diluted rat plasma in phosphate buffer, pH 7.4 (Jones, 1978). Each tachysterol metabolite was assessed at three different concentrations, curves were constructed, and the B_{50} value was calculated for each compound.

RESULTS

Metabolism of 25-OH-DHT_3 in Vitro. Perfusion of a kidney from a vitamin D replete rat for 6 h in the presence of 50 μg of 25-OH-DHT_3 resulted in the formation of at least seven further metabolites. Figure 1 illustrates a typical HPLC pattern on Zorbax-SIL of a 6-h perfusate extract; each metabolite is labeled (A–G inclusive). Each metabolite was purified by extensive HPLC and identified by the distinct DHT spectrum on diode array spectrophotometry with maxima at 240, 251, and 260 nm (Figure 2). Table I lists the retention times of these metabolites on HPLC on Zorbax-SIL and Zorbax-CN which ranged between those expected of a dihydroxylated derivative (cf. 24,25-dihydroxyvitamin D_3) and those expected of a trihydroxylated derivative (cf. 1,24,25-trihydroxyvitamin D_3). The retention times of analogous

Table I: Chromatographic Properties of Metabolites of 25-OH-DHT$_3$

peak designation	final identification	retention time on Zorbax-SIL[a] (min)	retention time on Zorbax-CN[b] (min)	retention time after periodate treatment[a] (min)
	25-OH-DHT$_3$	7.8	6.3	
A	24-oxo-25-OH-DHT$_3$	10.2	9.2	not changed
B	23,25-(OH)$_2$DHT$_3$	11.1	10.2	not changed
C	24-oxo-23,25-(OH)$_2$DHT$_3$	12.1	12.8	≈7.0[c]
D	24,25-(OH)$_2$DHT$_3$	14.0	10.5	≈8.0[c]
E	25-OH-DHT$_3$-26,23-lactone	15.5	18.9	not changed
F	25-OH-DHT$_3$-26,23-lactol	17.0[c]	14.7[c]	11.7
G	23,25,26-(OH)$_3$DHT$_3$	25.7	25.0	16.2
H[d]	unknown	20.3	17.0	not changed
K[d]	unknown	30.1	27.1	not changed
L[d]	unknown	32.0		
standards	25-OH-D$_3$	7.2	6.8	
	24,25-(OH)$_2$D$_3$	11.6	10.1	
	1,25-(OH)$_2$D$_3$	23.3	18.7	

[a] Zorbax-SIL; hexane–2-propanol–methanol, 91:7:2; 2 mL/min. [b] Zorbax-SIL; hexane–2-propanol–methanol, 94:5:1; 1.2 mL/min. [c] Broad peak. [d] Formed only in vivo.

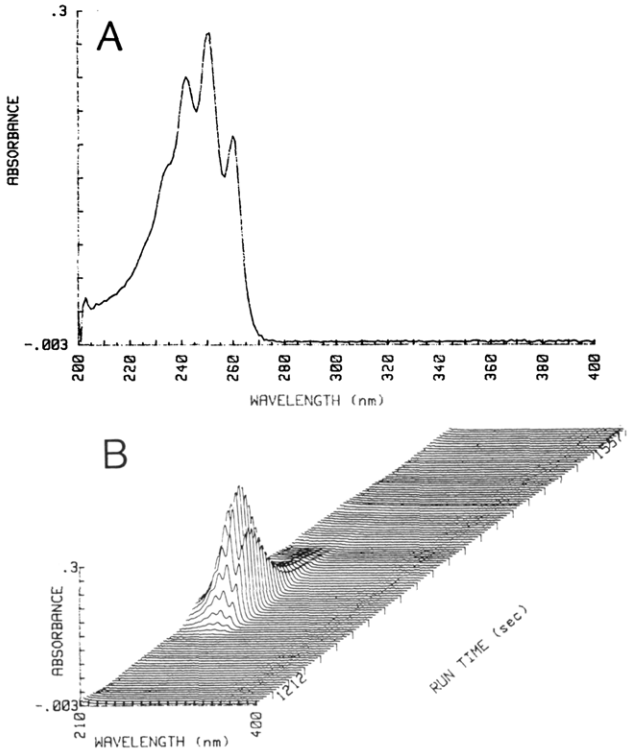

FIGURE 2: Typical UV spectra of a dihydrotachysterol. (A) Single scan of 24,25-(OH)$_2$DHT$_3$ depicting maxima at wavelengths of 242.5, 251, and 260.5 nm. (B) Diode array three-dimensional display of purified 24,25-(OH)$_2$DHT$_3$ emerging from final stages of HPLC. Axes: X = wavelength, Y = absorbance, and Z = time.

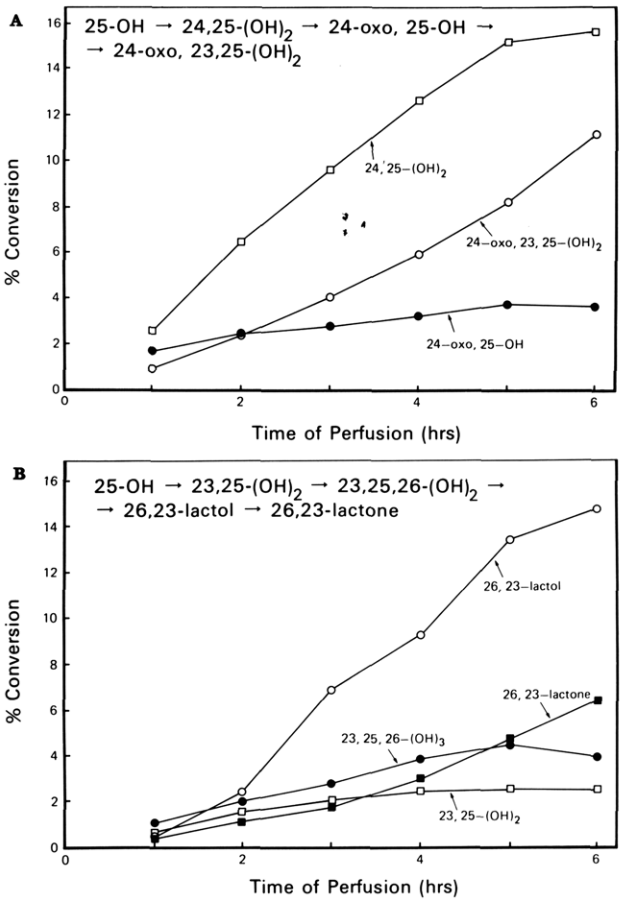

FIGURE 3: Time course of the appearance of metabolites of 25-OH-DHT$_3$ in the isolated perfused rat kidney. (A) Metabolism to 24,25-(OH)$_2$DHT$_3$ and its subsequent products. Symbols: (□) 24,25-(OH)$_2$DHT$_3$ (peak D); (●) 24-oxo-25-OH-DHT$_3$ (peak A); (○) 24-oxo-23,25-(OH)$_2$DHT$_3$ (peak C). (B) Metabolism involved in the pathway to 25-OH-DHT$_3$-26,23-lactone. Symbols: (□) 23,25-(OH)$_2$DHT$_3$ (peak B); (●) 23,25,26-(OH)$_3$D$_3$ (peak G); (○) 25-OH-DHT$_3$-26,23-lactol (peak F); (■) 25-OH-DHT$_3$-26,23-lactone (peak E). Data presented represent the mean of five perfusions. Coefficient of variation ≈ 10%.

derivatives of vitamin D$_3$ are, where possible, also included in Table I. One metabolite (F, Figure 1) gave a reproducible and highly characteristic broad peak during HPLC on both Zorbax-SIL and Zorbax-CN, suggesting the presence of some functionality other than a simple hydroxyl or oxo group. Three metabolites (A, C, and E) were more strongly retained on Zorbax-CN than the other metabolites, suggesting, by analogy with the behavior of metabolites of vitamin D$_3$ (Jones, 1983), that these compounds possess oxo groups.

Time Course of Appearance of in Vitro Metabolites. The kidney from a vitamin D replete rat synthesizes metabolites at a steady rate, with D representing the major product (Figure 3). Metabolite C was the only compound whose maximal rate of formation was significantly delayed, suggesting that it was distal to any other metabolites in any metabolic pathway. Figure 3 summarizes these data, illustrating the time course of formation of all seven metabolites, separated into two distinct metabolic pathways.

In contrast to the formation of these metabolites in kidneys from vitamin D replete rats, formation in the kidneys of vitamin D depleted rats was absent for the first 3 h of perfusion.

Table II: Induction of 25-OH-D_3-24-hydroxylase

inducer	concn	n	% conversion to 24,25-$(OH)_2D_3$[a]
ethanol control[b]		3	0.07
1α,25-$(OH)_2D_3$[c]	25 nM	4	1.46
1β,25-$(OH)_2D_3$[c]	25 nM	5	0.14
25-OH-DHT_3	25 nM	3	0.56
	100 nM	3	0.81
	1.25 μM	3	1.69

[a] Measured at 6 h after addition of inducer to an isolated kidney from a vitamin D deficient rat. [b] All inducers added in 100 μL of ethanol. [c] 1α,25-$(OH)_2D_3$, positive control; 1β,25-$(OH)_2D_3$, negative control.

This suggests that the enzymes required for the biosynthesis of the seven identified metabolites are absent in the vitamin D depleted animal and are induced during the initial 3-h perfusion. In the 3–6-h perfusion period, synthesis of the same seven metabolites occurs, but the total amounts accumulating over the 6-h perfusion are less than in the kidney from the vitamin D replete rat.

The effect of various added compounds, including 25-OH-DHT_3, on the conversion of radiolabeled 25-OH-D_3 to either 1,25-$(OH)_2D_3$ or 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$] was examined in kidneys from the vitamin D depleted rat. The results of these experiments are summarized in Table II. It is clear that 25-OH-DHT_3, particularly at high concentrations, was able to induce the 24-hydroxylase enzyme.

Further in vitro studies were carried out with rat kidney mitochondria and chick kidney homogenates from vitamin D deprived rats. Incubation of 25-OH-DHT_3 with both these preparations did not give rise to detectable quantities of peak H, which had been observed in substantial amounts in in vivo experiments. In both these in vitro experiments [^{3}H]-25-OH-D_3 was converted in good yield to [^{3}H]-1,25-$(OH)_2D_3$.

Structural Identification of Metabolites Formed in Kidneys from Vitamin D Replete Rats. (Peak A) 24-Oxo-25-hydroxydihydrotachysterol$_3$ (24-Oxo-25-OH-DHT_3). Peak A was the least polar metabolite with a retention time of 10.9 min on Zorbax-SIL HPLC and was more strongly retained on Zorbax-CN, similarly to peak C (see Table I). This suggested the presence of an oxo group in peak A. The metabolite was insensitive to periodate oxidation, suggesting the absence of vicinal oxygen functions. Direct probe mass spectrometry of the underivatized compound gave a molecular ion at m/z 416, suggesting the addition of one oxygen to the parent molecule 25-OH-DHT_3 (M_r 402), together with the loss of two hydrogens. Mass fragments representing loss of side chain (m/z 273) and loss of side chain and dehydration at C3 (m/z 255) were also present. An abundant peak (m/z 121) in all the metabolites represents the A-ring – H_2O after cleavage between C7 and C8. A major fragment was also seen at m/z 358, which is probably derived by the loss of 58 mass units by cleavage between C24 and C25, seen in the mass spectra of C24-substituted vitamin D metabolites (Jones et al., 1979, 1980). This evidence suggests that the additional substitution in this metabolite is at C24 and is probably an oxo group. Peak A also formed a methyl oxime, trimethylsilyl derivative in small yield which after GC–MS gave a mass spectrum with a molecular ion at m/z 589 and major fragments at m/z 560 [(M – oxime)$^+$], 499 ([M – 90)$^+$], and 131 [(C24,26,25-OTMSi fragment)$^+$]. GC–MS of the per(trimethylsilyl) ether derivative gave a molecular ion of m/z 560 with major mass fragments at m/z 545 [(M – 15)$^+$] and 131. Other fragments were seen at m/z 255 and 429 [(M – 131)$^+$]. All the data are consistent with the assignment of the structure of peak A as 24-oxo-25-OH-DHT_3.

(Peak B) 23,25-Dihydroxydihydrotachysterol$_3$ [23,25-(OH)$_2$DHT$_3$]. Peak B had a retention time of 12.0 min on Zorbax-SIL and did not change its position relative to that of peak D on Zorbax-CN chromatography (see Table I). The metabolite was insensitive to periodate oxidation, again indicating that vicinal oxygen functions were not present. Direct probe mass spectrometry of the underivatized compound gave a molecular ion at m/z 418, suggesting the addition of an extra hydroxyl group to 25-OH-DHT_3. The presence of mass fragments at m/z 273 [(M – side chain)$^+$] and 255 [M – side chain – H_2O)$^+$] suggested that the extra hydroxyl was on the side chain. The substitution must therefore have occurred on positions other than C24, C25, C26, or C27. A minor fragment at m/z 344 was interpreted as representing the fragment obtained after loss of part of the side chain, cleaved between C23 and C24. An analogous fragment was observed in the mass spectrum of 23,25-dihydroxyvitamin D_3 [23,25-$(OH)_2D_3$] by Napoli et al. (1982). The mass spectrum obtained from GC–MS of the per(trimethylsilyl) ether derivative was consistent with a structure of 23,25-$(OH)_2DHT_3$. GC–MS of the per(trimethylsilyl) ether derivative of $^2H_6$-labeled peak B gave a molecular ion at m/z 640 and fragments at m/z 625 [(M – 15)$^+$], 550 [(M – 90)$^+$], 460 [(M – 90 – 90)$^+$], and 137 [(hexadeuteriated C26-, C27-, and C25-OTMSi)$^+$]. The fragility of the C23–C24 bond and other spectral data suggest but do not prove that peak B is 23,25-$(OH)_2DHT_3$.

(Peak C) 24-Oxo-23,25-dihydroxydihydrotachysterol$_3$ [24-Oxo-23,25-(OH)$_2$DHT$_3$]. Peak C had a retention time of 12.4 min on Zorbax-SIL HPLC, eluting just ahead of peak D. On Zorbax-CN, the order of elution was reversed, D eluting before C (see Table I). This suggests that peak A contains a >C=O function. In kinetic studies, the accumulation of peak C was noticeably delayed over that of other metabolites, suggesting that its formation occurred later in a metabolic pathway than the other metabolites. Direct probe mass spectrometry showed a molecular ion at m/z 432, indicating the addition of two oxygen functions and loss of two hydrogens, suggesting the presence of an extra hydroxyl and an oxo group. The molecule was sensitive to periodate oxidation, suggesting the presence of vicinal oxygen functions. The presence of other fragments at m/z 273, 255, and 121 suggested that the extra oxygen functions were on the side chain. A further fragment was seen at m/z 374 which was interpreted as being derived by loss of C26-, C27-, and C25-OH, indicating that the additional oxygen functions were on the side chain between C20 and C24. GC–MS of the per(trimethylsilyl) ether metabolite gave a molecular ion at m/z 648 indicating the additions of three trimethylsilyl groups. Key fragments in this spectrum were seen at m/z 489, 399, and 309 which were interpreted as being derived by cleavage between C23 and C24, the fragment m/z 489 retaining two trimethylsilyl groups which are then sequentially lost to give m/z 399 and 309. Again this fragility between C23 and C24 suggests the presence of an oxygen function at C23, and an analogous fragment is seen in 24-oxo-23,25-dihydroxyvitamin D_3 (Napoli & Horst, 1983) and in other 23-hydroxylated vitamin D_3 metabolites (Napoli et al., 1982; Wichmann et al., 1981). These fragments are also seen in peak G which has been assigned the structure 23,25,26-trimethylsilyl-DHT_3. An alternative structure, 23-oxo-24,25-$(OH)_2DHT_3$, can be ruled out, since cleavage of the C23–C24 bond would have yielded a fragment containing only one trimethylsilyl group. Peak C formed in very low yield an oxime, trimethylsilyl ether derivative with a molecular ion at m/z 677, indicating the presence of a single oxo group. $^2H_6$-labeled peak B pertri-

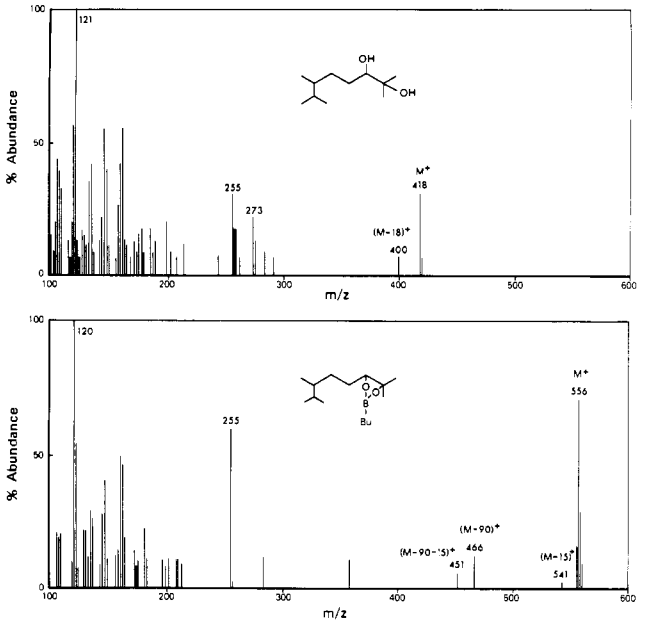

FIGURE 4: Positive ion EI mass spectra of peak D [24,25-$(OH)_2DHT_3$] isolated from kidney perfusate. Spectra were obtained from the underivatized compound by direct probe insertion (upper trace) or after formation of the *n*-butylboronate–trimethylsilyl ether derivative by scanning the maximum of the chromatographic peak obtained during GC–MS.

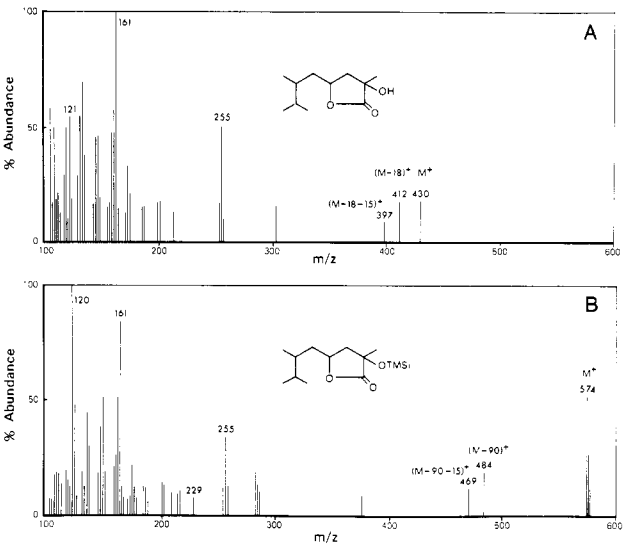

FIGURE 5: Positive ion EI mass spectra of peak E (25-OH-DHT_3-26,23-lactone). Spectra were obtained, by direct probe insertion, from the underivatized compound isolated from kidney perfusate (upper trace) and from the per(trimethylsilyl) ether derivative of the compound isolated from rat plasma, by scanning the maximum of the chromatographic peak obtained during GC–MS (lower trace). A similar spectrum was obtained from the per(trimethylsilyl) ether derivative of peak E isolated from kidney perfusate.

methylsilyl ether derivative had the expected molecular ion at m/z 654 and C23–C24 cleavage fragments at m/z 489, 399, and 309. The fact that these C23–C24 cleavage fragments in the hexadeuteriated metabolite have the same mass/charge ratio as fragments from the nondeuteriated metabolite confirms that these fragments are indeed derived by loss of part of the side chain. It is concluded therefore that peak B is 24-oxo-23,25-$(OH)_2DHT_3$.

(*Peak D*) *24,25-Dihydroxydihydrotachysterol*$_3$ [*24,25-(OH)$_2$DHT$_3$*]. Peak D is the major metabolite of 25-OH-DHT_3 in vitro with a retention time of 15.0 min on Zorbax-SIL HPLC, slightly more polar than 24,25-$(OH)_2D_3$ (see Table I). Direct probe mass spectrometry of the underivatized compound (Figure 4) gave a molecular ion at m/z 418 with fragments at m/z 273, 255, and 121, commonly seen in metabolites of DHT_3. These fragments indicate that any substitution of 25-OH-DHT_3 has occurred in the side chain, and the increase of 16 mass units probably represents an extra hydroxyl group. Peak D is susceptible to periodate oxidation, giving rise to a product which elutes as a broad peak on Zorbax-SIL HPLC, similar in chromatographic properties to the periodate cleavage product from 24,25-$(OH)_2D_3$. GC–MS of the per(trimethylsilyl) ether derivative gave a molecular ion at m/z 634, with mass fragments at m/z 619 [$(M - 15)^+$], 544 [$(M - 90)^+$], 454 [$(M - 90 - 90)^+$], 413 [$(M - 131 - 90)^+$], 255, and 131. An *n*-butylboronate cyclic ester–trimethylsilyl ether derivative could also be formed, giving a mass spectrum with a molecular ion at m/z 556 and mass fragments at m/z 541 [$(M - 15)^+$], 466 [$(M - 90)^+$], 451 [$(M - 90 - 15)^+$], 255, and 120 [(A-ring $- 90)^+$]. The per(trimethylsilyl) $^2H_6$-labeled metabolite gave a molecular ion at m/z 640 and a fragment at m/z 137. All the data are consistent with the assignment of the structure of peak D as 24,25-$(OH)_2DHT_3$.

(*Peak E*) *25-Hydroxydihydrotachysterol*$_3$ *26,23-Lactone* (*25-OH-DHT$_3$-26,23-lactone*). Peak E has a retention time of 15.8 min on Zorbax-SIL HPLC, slightly more polar than peak D. On Zorbax-CN however, peak E is retained, eluting several minutres later than peak D (see Table I). This chromatographic behavior suggests the presence of a >C=O function. Periodate oxidation had no effect on peak E. Direct probe mass spectrometry (Figure 5A) gave a molecular ion at m/z 430, an increase of 28 mass units over that of 25-OH-DHT_3, probably representing the addition of two oxygen functions. Mass fragments at m/z 273 and 255 were present indicating that the substitution had probably occurred in the side chain. It was suspected from the chromatographic properties of this metabolite that it might be the lactone, and the mass spectrum of 25-hydroxyvitamin D_3 26,23-lactone (25-OH-D_3-26,23-lactone) was very similar to that obtained from peak E (Wichmann et al., 1979). GC–MS of the per(trimethylsilyl) ether derivative gave a molecular ion at m/z 574, indicating that the two extra oxygen functions on the side chain are not hydroxyls. Similarly to the spectrum of 25-OH-D_3-26,23-lactone, no fragment at m/z 131 was seen. This characteristic fragment arises by cleavage between C24 and C25, giving a C26,C27,C25-OTMSi usually seen in the mass spectrum of 25-hydroxylated derivatives. Other mass fragments at m/z 484 [$(M - 90)^+$], 469 [$(M - 90 - 15)^+$], 255, and 120 (A-ring $- 90)^+$] were also present. Peak E was also formed in vivo and accumulated in the plasma of rats given DHT_3. The chromatographic properties of peak E derived from rat plasma were identical with those of in vitro formed peak E, and the mass spectra were identical. Peak E has therefore been assigned the structure of 25-OH-DHT_3-26,23-lactone.

(*Peak F*) *25-Hydroxydihydrotachysterol*$_3$ *26,23-Lactol* (*25-OH-DHT$_3$-26,23-lactol*). Peak F elutes as a broad tailing peak in both Zorbax-SIL and Zorbax-CN HPLC systems. The retention time of peak F on Zorbax-SIL is similar to that of 25,26-dihydroxyvitamin D_3 [25,26-$(OH)_2D_3$] and thus suggests that this compound contains three oxygen functions (see Table I). Peak F is susceptible to periodate oxidation, and the oxidation product elutes as a sharp peak with a retention time similar to that of peak B on Zorbax-SIL HPLC. This suggests the presence of vicinal oxygen groups, possibly on either C24 or C26 (C27). Direct probe mass spectrometry of the underivatized compound (Figure 6) gave a molecular ion at m/z 432, the same as peak C to which the structure of 24-oxo-23,25-$(OH)_2DHT_3$ has been assigned. However,

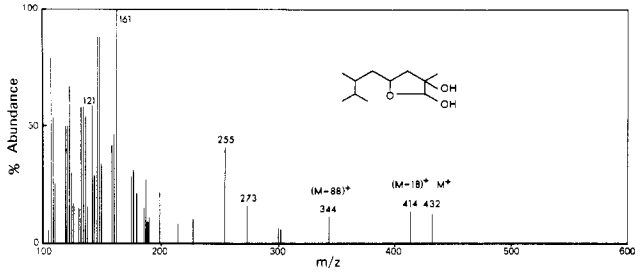

FIGURE 6: Positive ion EI mass spectrum of peak F (25-OH-DHT_3-26,23-lactol) isolated from kidney perfusate, obtained by direct probe insertion of the underivatized compound.

the fragmentation pattern of peak F differed significantly from that seen for peak C, giving an intense fragment at m/z 344, which is interpreted as loss of side chain cleaved between C23 and C24, but no fragment at m/z 374, present in the spectrum of peak C, was seen. Mass fragments were also present at m/z 273, 255, and 121. The C23–C24 cleavage suggests the presence of an oxygen function at C23. It was observed that the mass spectrum of the underivatized compound was very similar to that reported by Yamada et al. (1984) for 25-hydroxyvitamin D_3 26,23-lactol (25-OH-D_3-26,23-lactol) which gave mass fragments at m/z 342 [(loss of side chain by C23–C24 cleavage)$^+$], 271 [(loss of side chain by C17-20 cleavage)$^+$], and 253 [(271 − 18)$^+$]. The putative 25-OH-DHT_3-26,23-lactol contains vicinal hydroxyl groups at C25 and C26 which can thus be cleaved by periodate, and the structure is also susceptible to C23–C24 cleavage during mass spectrometry. The per(trimethylsilyl) ether derivative when subjected to GC–MS gave a molecular ion at m/z 648, indicating the incorporation of three trimethylsilyl groups, presumably at C3, C25, and C26. Similarly to the analogous vitamin D lactol, GC–MS of the per(trimethylsilyl) ether derivative of peak C gave rise to a molecular ion at m/z 648 and to a mass fragment at m/z 530, which arises by loss of 118 mass units (OCH − OTMSi), representing the cleavage of the lactol ring between C23–O and C25–C26. The per-(trimethylsilyl) ether derivative of deuteriated peak F derived from [$^2H_6$]-25-OH-DHT_3 gave a molecular ion at m/z 652, indicating that during the formation of peak F two deuterium atoms had been lost from C26 or C27. This loss of deuterium atoms is consistent with a lactol structure. It is therefore concluded that peak F has the structure 25-OH-DHT_3-26,23-lactol.

Yamada et al. (1984) have suggested that 25-OH-D_3-26,23-lactol is an intermediate in the biosynthesis of the 25-OH-D_3-26,23-lactone. The identification of peak E as 25-OH-DHT_3-26,23-lactone adds therefore additional support to the suggestion that peak F is the 25-OH-DHT_3-26,23-lactol.

(Peak G) 23,25,26-Trihydroxydihydrotachysterol$_3$ [23,25,26-(OH)$_3$DHT$_3$]. Peak G is the most polar metabolite of 25-OH-DHT_3 formed in the perfused kidney which has been isolated to date. It has a retention time on Zorbax-SIL of 35 min and is thus more polar than 1,25-$(OH)_2D_3$ (see Table I). Although the peak was broad, it is suggested that this is due mainly to the long retention time rather than any peculiar functionality. Increasing solvent strength (to hexane–2-propanol–methanol, 88:10:2 v/v/v, for example) decreased the retention time of peak F and sharpened the peak. Peak G was susceptible to periodate oxidation and gave rise to a product that had similar chromatographic properties to those of peak B. Because of poor yields of peak G, no direct probe mass spectrometry of the underivatized compound was carried out. GC–MS of the per(trimethylsilyl) ether derivative gave a molecular ion at m/z 722, indicating the incorporation of four trimethylsilyl groups into presumably four hydroxyl groups. Mass fragments were also observed at m/z 632 (M − 90), 542 (M − 90 − 90), 452 (M − 90 − 90 − 90), and 362 (M − 90 − 90 − 90 − 90). A number of other mass fragments attributable to loss of 233 mass units (by cleavage between C23 and C24 and subsequent loss of two silanol groups) were also observed at m/z 489, 399, and 309. Cleavage also occurred between C24 and C25, giving fragments at m/z 219 and 129 (219 − 90). This fragmentation suggests the presence of a hydroxyl group at C23 of fragmentation observed in mass spectrum of peak C and at C26 (or C27), suggesting that the structure of peak G is 23,25,26-$(OH)_3DHT_3$. Thus, peak G would be the metabolic precursor of peaks E and F.

Table III: Binding Properties of Certain Tachysterol Metabolites

name	binding to rat DBP[a]
25-OH-DHT_3	900
24-oxo-25-OH-DHT_3	502
24-oxo-23,25-$(OH)_2DHT_3$	>10 000
24,25-$(OH)_2DHT_3$	559
25-OH-DHT_3-26,23-lactone	205

[a] B_{50} relative to a value of 1 for 25-OH-D_3. B_{50} for 25-OH-D_3 = 250 pg/tube.

In Vivo Metabolism of DHT_3. A single injection of DHT_3 resulted in the production of a number of in vivo metabolites, many of which were only present in small quantities. Each compound was identified as a metabolite of DHT_3 by its characteristic UV spectrum, obtained by photodiode array spectrophotometry. When an extract from rat plasma, obtained 18 h after administration of DHT_3, was chromatographed on straight-phase Zorbax-SIL, as illustrated in Figure 7 (A and B), a number of peaks with typical DHT spectra were observed. Only four of these peaks could be identified as having chromatographic mobilities identical with those of peaks obtained from the perfused kidney. These were 25-OH-DHT_3, 24-oxo-25-OH-DHT_3 (peak A), peak D [24,25-$(OH)_2DHT_3$], and peak E (25-OH-DHT_3-26,23-lactone). In the case of peak E the identification as 25-OH-DHT_3-26,23-lactone was confirmed by mass spectrometry of the rat plasma metabolite (Figure 5B). Four other major peaks seen in vitro, peaks B, C, F, and G, were not observed in significant quantities in the in vivo experiments. Figure 7 (A and B) indicates that after a single dose of DHT_3 the 25-OH-DHT_3-26,23-lactone accumulates in greater concentrations than the 24,25-$(OH)_2DHT_3$ metabolite, but the metabolite present in greatest concentrations under these conditions is peak H. At the moment these more polar in vivo metabolites have not been identified.

Binding Studies. Limited binding studies using rat plasma DBP have been carried out with the metabolites isolated from the perfused kidney. Results are summarized in Table III. All compounds were relatively ineffective at displacing 25-OH-D_3 from the vitamin D transport protein.

Discussion

This paper reports the isolation and identification of seven side chain modified metabolites of 25-OH-DHT_3 formed in vitro by the perfused rat kidney. All seven metabolites are analogous to previously identified metabolites of vitamin D_3 and can be placed on two separate pathways of renal metabolism which are illustrated in Figure 8. Although more polar metabolites of DHT_3 were mentioned by Hallick and DeLuca (1972), Seymour and DeLuca (1974), and Lawson and Bell (1974), they were not isolated or identified. The work described here therefore represents the first comprehensive study of the renal metabolism of DHT_3. The time course studies

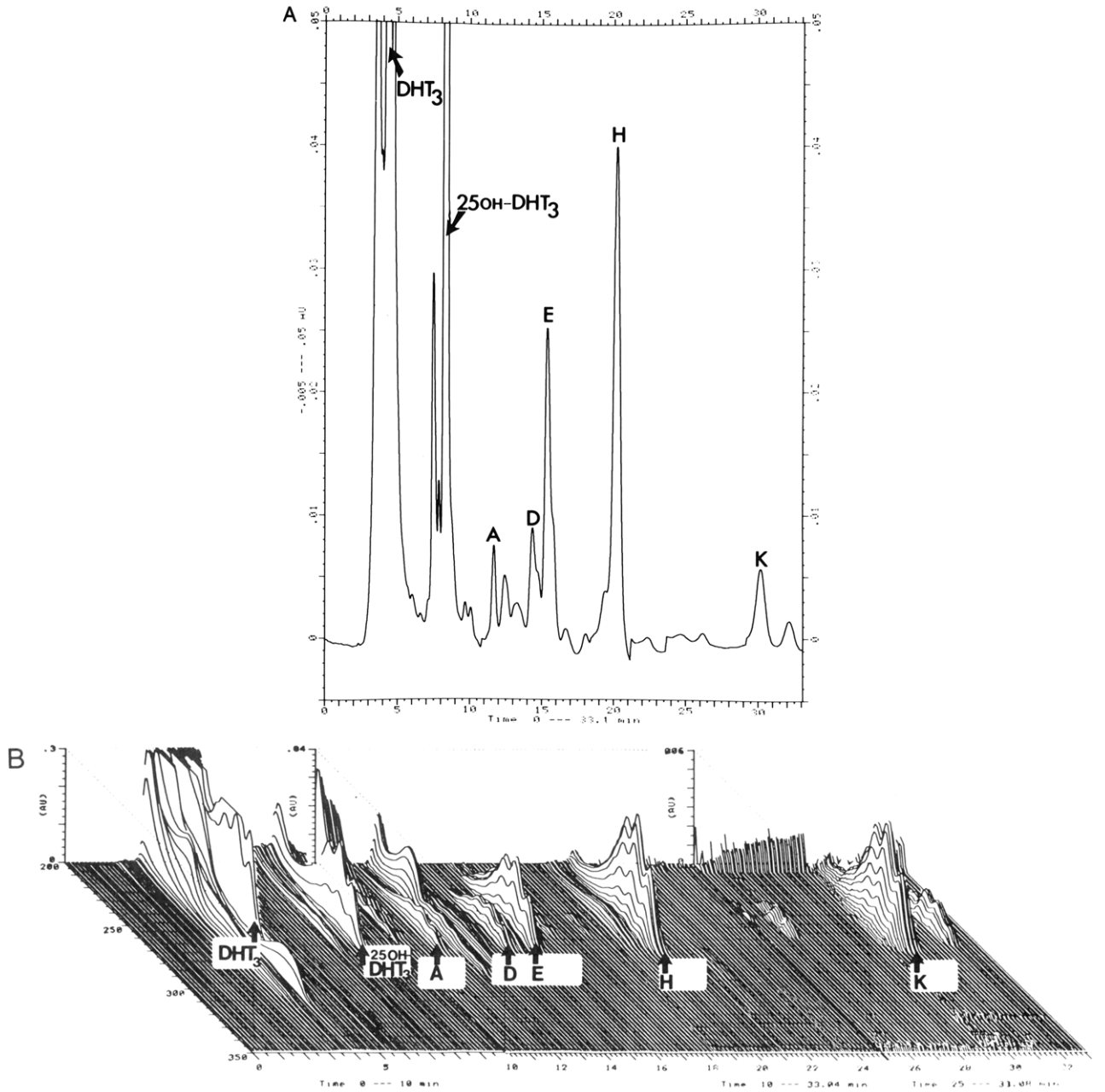

FIGURE 7: HPLC of lipid extract of plasma from rats given a single injection of DHT_3 18 h previously. (A) Single-wavelength (251 nm) detection. (B) Diode array spectrophotometric detection. Conditions used were as follows: Zorbax-SIL (0.62 × 25 cm); hexane–2-propanol–methanol (91:7:2 v/v/v); 2 mL/min. Metabolites are labeled A–L. The metabolite peaks A, D, and E were given the same letter designations as metabolites observed in kidney perfusions on the basis of their retention on HPLC. In the case of peak E, subsequent identification by MS confirmed this letter designation.

in the perfused kidney support the sequence of intermediates of both pathways illustrated in Figure 8. Although the identification of these metabolites is not unequivocal, there is considerable evidence that the assigned structures are probably correct. The fact that the seven metabolites can be sequentially placed in metabolic pathways already established for vitamin D gives added weight to their identification. The enzymes responsible for the synthesis of the seven side chain modified metabolites of 25-OH-DHT_3 are absent in the vitamin D deficient rat and are induced by either dietary vitamin D supplementation or treatment of the kidney with large amounts of 25-OH-DHT_3. This implies these enzymes are the same as those responsible for the side-chain modification of 25-OH-D_3.

Although not all the metabolites found in the perfused rat kidney were detected in rat plasma after injection of DHT_3, the 25-OH-DHT_3-26,23-lactone which is the final product of one of the pathways illustrated in Figure 8 and 24,25-$(OH)_2DHT_3$ which is probably, by analogy with vitamin D_3 metabolism, a major metabolic intermediate in the excretion of the molecule were seen. The replacement of the C10–C19 methylene group with a C19 methyl group and rotation of the A-ring in 25-OH-DHT_3 do not seem to be very important for substrate binding to side chain hydroxylating enzymes since both DHT_3 and vitamin D_3 are qualitatively metabolized by the same pathways. The demonstration of the presence of 24-oxo-25-OH-DHT_3 and 24-oxo-23,25-$(OH)_2DHT_3$ indicates that the 24-oxidation pathway proposed for vitamin D_3 may also be operative for the metabolism of the side chain of DHT_3 (Jones et al., 1983, 1984; Reddy et al., 1987).

The abundance of metabolites of 25-hydroxy-DHT_3 involved as intermediates in the formation of the 26,23-lactone which were observed in the perfused kidney adds credence to the postulated metabolic pathway of formation of 25-OH-D_3-

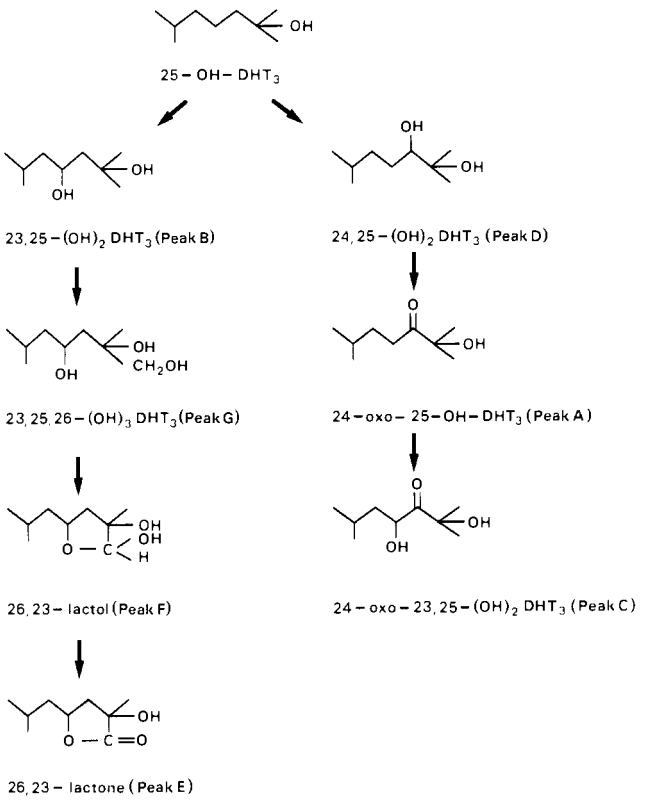

FIGURE 8: Proposed pathways of 25-OH-DHT_3 metabolism.

26,23-lactone (Yamada et al., 1984). Most of these intermediates in the biosynthesis of 25-OH-D_3-26,23-lactone have been identified individually, and the system described here is remarkable in that all the postulated intermediate compounds have been identified in a single system which thus represents an ideal model for the study of this metabolic sequence. The presence of such large amounts of 25-OH-DHT_3-26,23-lactone in vivo and in vitro is difficult to explain since the physiological function of 25-OH-D_3-26,23-lactone is still unclear (Ishizuka et al., 1984). On the other hand, the difference in DBP binding affinities for the various tachysterol metabolites (Table III) may also play some role in the nature of the metabolites found to be circulating in vivo. It is probably no coincidence that DBP has the greatest affinity for 25-OH-D_3-26,23-lactone and this metabolite accumulated in large amounts in vivo.

The demonstration of the formation in vivo of more polar metabolites of DHT_3 (peaks H, K, and L) which are apparently not formed by the perfused kidney in vitro nor by incubation of 25-OH-DHT_3 with homogenates or mitochondria from kidneys from vitamin D deprived rats suggests that these compounds are extrarenal in origin. This observation may be important because DHT is known to be biologically potent in anephric animals (Harrison & Harrison, 1972) where renal metabolites would be absent. Peak H which has similar chromatographic mobility to 1,25-$(OH)_2D_3$ may be similar to the metabolite(s) isolated by Bosch et al. (1985a,b) and suggested to be 1,25-$(OH)_2DHT_2$. Furthermore, recent preliminary data from our laboratories (Porteous et al., 1988) suggest that peak H is only 10 times less effective than 1,25-$(OH)_2D_3$ at displacing [^{3}H]-1,25-$(OH)_2D_3$ from chick intestinal receptor, a fact that makes it a candidate for the calcemic factor of DHT. The fact that peak H is *not* made from 25-OH-DHT_3 in the kidney suggests that if peak H is in fact 1-hydroxylated, and this is by no means proved, the enzyme responsible for this hydroxylation must be extrarenal. Although extrarenal 25-hydroxyvitamin D_3 1α-hydroxylase has been found in several sites in vitro (Howard et al., 1981; Reichel et al., 1987), it has only been found in vivo in the placenta (Gray & Lester, 1980) and sarcoid macrophage (Adams et al., 1984) so that the exact location of this putative DHT-hydroxylase remains obscure. Gray et al. (1979) have also demonstrated in plasma of anephric patients treated with DHT_2 the presence of a putative metabolite which comigrated with authentic [^{3}H]-1,25-dihydroxyvitamin D_3 on HPLC and suggested the possibility that the biological effects of DHT_2 might be mediated via this metabolite.

In conclusion, it is clear that DHT undergoes extensive metabolic modification in vivo before it is cleared from the mammalian body. Our results suggest that the kidney represents a major, but not the only, site of synthesis of these metabolites. It will be interesting to determine which of this multitude of products will turn out to be the calcemic factor observed in clinical studies.

ACKNOWLEDGMENTS

Dr. K. Nakatsu, Department of Pharmacology, Queen's University, operated the mass spectrometer used in some of the studies reported here. We thank Drs. Michael and Sally Holick, Tuft's University, Boston, for the generous gift of 1β,25-dihydroxyvitamin D_3 used in the renal 24-hydroxylase induction studies.

Registry No. 25-OH-DHT_3, 25631-39-4; 23,25-$(OH)_2DHT_3$, 115563-29-6; 24,25-$(OH)_2DHT_3$, 115563-30-9; 23,25,26-$(OH)_3DHT_3$, 115563-31-0; 24-oxo-25-OH-DHT_3, 115563-32-1; 24-oxo-23,25-$(OH)_2DHT_3$, 115563-33-2; 25-OH-DHT_3-26,23-lactone, 115591-07-6; 25-OH-DHT_3-26,23-lactol, 115591-08-7; 25-OH-D_3-24-hydroxylase, 53112-53-1.

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Inhibition of Serine Palmitoyltransferase in Vitro and Long-Chain Base Biosynthesis in Intact Chinese Hamster Ovary Cells by β-Chloroalanine†

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ABSTRACT: The effects of β-chloroalanine (β-Cl-alanine) on serine palmitoyltransferase activity and the de novo biosynthesis of sphinganine and sphingenine were investigated in vitro with rat liver microsomes and in vivo with intact Chinese hamster ovary (CHO) cells. The inhibition in vitro was rapid (5 mM β-Cl-alanine caused complete inactivation in 10 min), irreversible, and concentration and time dependent and apparently involved the active site because inactivation only occurred with β-Cl-L-alanine (not β-Cl-D-alanine) and was blocked by L-serine. These are characteristics of mechanism-based ("suicide") inhibition. Serine palmitoyltransferase (SPT) was also inhibited when intact CHO cells were incubated with β-Cl-alanine (complete inhibition occurred in 15 min with 5 mM), and this treatment inhibited [^{14}C]serine incorporation into long-chain bases by intact cells. The concentration dependence of the loss of SPT activity and of long-chain base synthesis was identical. The effects of β-Cl-L-alanine appeared to occur with little perturbation of other cell functions: the cells exhibited no loss in cell viability, [^{14}C]serine uptake was not blocked, total lipid biosynthesis from [^{14}C]acetic acid was not decreased (nor was the appearance of radiolabel in cholesterol and phosphatidylcholine), and [^{3}H]thymidine incorporation into DNA was not affected. There appeared to be little effect on protein synthesis based on the incorporation of [^{3}H]leucine, which was only decreased by 14%. Although β-Cl-L-alanine is known to inhibit other pyridoxal 5′-phosphate dependent enzymes, alanine and aspartate transaminases were not inhibited under these conditions. These results establish the close association between the activity of serine palmitoyltransferase and the cellular rate of long-chain base formation and indicate that β-Cl-alanine and other mechanism-based inhibitors might be useful to study alterations in cellular long-chain base synthesis.

Serine palmitoyltransferase (SPT) (EC 2.3.1.50) is a pyridoxal 5′-phosphate dependent enzyme that catalyzes the first committed step of sphingolipid biosynthesis, the condensation of palmitoyl-CoA and serine to form 3-ketosphinganine (Snell et al., 1970). Several lines of evidence suggest that SPT catalyzes a rate-limiting step in sphingolipid biosynthesis (Braun et al., 1970; Williams et al., 1984b). SPT appears to

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