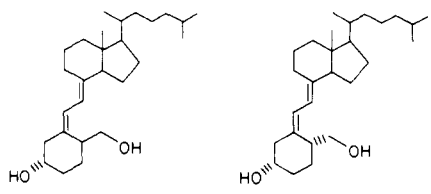
In Vivo and in Vitro Inhibition of Rat Liver Vitamin D_3-25-hydroxylase Activity by 19-Hydroxy-10(*S*),19-dihydrovitamin D_3†

Herbert E. Paaren, Robert M. Moriarty, Heinrich K. Schnoes, and Hector F. DeLuca*

ABSTRACT: Rats treated with varying amounts of 19-hydroxy-10(*S*),19-dihydrovitamin D_3 prior to administration of physiologic doses of vitamin D_3 exhibit normal intestinal calcium transport but are unable to mobilize bone calcium. In contrast, 19-hydroxy-10(*R*),19-dihydrovitamin D_3 had no inhibitory activity. Circulating serum levels of 25-hydroxy-[^{3}H]vitamin D_3 and 1α,25-dihydroxy[^{3}H]vitamin D_3 are markedly suppressed but not totally eliminated in animals predosed with 19-hydroxy-10(*S*),19-dihydrovitamin D_3 before [^{3}H]vitamin D_3. Hepatic 25-hydroxy[^{3}H]vitamin D_3 levels were approximately equal in both 19-hydroxy-10(*S*),19-dihydrovitamin D_3 treated and untreated rats. However, the rate of conversion of [^{3}H]vitamin D_3 to 25-hydroxyvitamin D_3 in vivo is greatly reduced in the treated rats. The inhibitory vitamin analogue was also shown to block hepatic microsomal 25-hydroxylation in vitro. These results indicate that 19-hydroxy-10(*S*),19-dihydrovitamin D_3 is a specific inhibitor for a hepatic microsomal vitamin D_3-25-hydroxylase system.

The primary step in the metabolic functionalization of vitamin D_3, 25-hydroxylation, is a unique control point in overall expression of vitamin D activity since all known biologically active vitamin D metabolites are produced from 25-hydroxyvitamin D_3 (25-OH-D_3)[1] (DeLuca et al., 1979). The liver is believed to be the site of 25-hydroxylation although extrahepatic 25-hydroxylase activity has been reported (Tucker, 1973; Bhattacharyya & DeLuca, 1974a; Holick et al., 1976). Hepatic 25-hydroxylase activity has been found in both the microsomal (Bhattacharyya & DeLuca, 1974b; Bjorkhem et al., 1979; Sulimovici et al., 1979) and mitochondrial (Bjorkehem & Holmberg, 1978) fractions. However, the microsomal system is considered to be the major site for this conversion (Madhok & DeLuca, 1979). Inasmuch as 25-OH-D_3 can have biological activity without 1-hydroxylation when given in large amounts, the most effective antagonist of vitamin D activity should inhibit prior to 25-hydroxylation. The side-chain analogue of vitamin D_3, 25-azavitamin D_3, is known to be an effective antagonist of vitamin D activity because of its ability to block 25-hydroxylation in the rat (Onisko et al., 1979a). Other side-chain analogues such as 25-fluorovitamin D_3, 24,25-dehydrovitamin D_3, and 25,26-dehydrovitamin D_3 are effective 25-hydroxylase inhibitors, but they do not block vitamin D activity because they themselves are metabolized to biologically active vitamin D derivatives (Onisko et al., 1979b). The A-ring-modified analogue 19-hydroxy-10(*S*),19-dihydrovitamin D_3 [19-OH-10(*S*),19-DHD$_3$], which

19-HYDROXY-10(19)-DIHYDROVITAMIN D_3



10S 10R

was independently synthesized from (19*E*)-acetoxyvitamin D_3 acetate via $NaBH_4$ reduction (Paaren, 1976) or directly from vitamin D_3 by the action of 9-borobicyclononane and basic hydrogen peroxide (Mourino & Okamura, 1978), has been reported to antagonize vitamin D activity in chicks by blocking 25-hydroxylase activity (Norman et al., 1977).

The work presented in this report deals with the inhibitory properties of 19-OH-10(*S*),19-DHD$_3$ in the rat. The in vivo and in vitro results herein suggest that it is a highly active inhibitor for the specific microsomal vitamin D_3-25-hydroxylase systems.

Materials and Methods

Vitamin D_3 Compounds. 25-Hydroxyvitamin D_3 was a gift from the Upjohn Co. (Kalamazoo, MI). Vitamin D_3 was purchased from Phillips-Duphar Co. (Weesp, The Netherlands). (*10R*)- and (*10S*)-19-hydroxy-10,19-dihydrovitamin D_3 were synthesized according to the method of Mourino & Okamura (1978). [3α-^{3}H]Vitamin D_3 was synthesized according to the method of S. Yamada, H. K. Schnoes, and H. F. DeLuca (unpublished results).

Animals. Male albino weanling rats (Holtzman Co., Madison, WI) were housed in hanging wire cages and fed ad libitum a vitamin D deficient diet for 3 weeks followed by an additional 2 weeks on a vitamin D deficient, low calcium diet before use in bioassays. Male albino weanling rats housed as above were maintained on a vitamin D deficient, low calcium diet for 3 weeks before use in the metabolism studies.

Biological Assays. Vitamin D and calcium deficient rats, numbering six in each group unless otherwise noted, received the first dose of 50 μL of ethanol and either 19-OH-10(*R*),19-DHD$_3$ or 19-OH-10(*S*),19-DHD$_3$ intrajugularly under light ether anesthesia followed by a second dose of 50 μL of ethanol and either vitamin D_3 or 25-OH-D_3 1, 2, 6, 8, or 10 h later by the same route of administration. All compounds were given in 50 μL of ethanol. Between 20 and 22 h after the second dose, the animals were killed by decapitation. Bone calcium mobilization was determined by measuring serum calcium levels; 0.1-mL aliquots of serum were diluted with 1.9 mL of 0.1% aqueous $LaCl_3$, and calcium was measured by atomic absorption spectroscopy (Perkin-Elmer Model 412 atomic absorption spectrometer). Duodenal calcium transport was determined by the everted gut sac technique of Martin

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[1] Abbreviations used: 25-OH-D_3, 25-hydroxyvitamin D_3; 19-OH-10(*S*),19-DHD$_3$, 19-hydroxy-10(*S*),19-dihydrovitamin D_3; 19-OH-10(*R*),19-DHD$_3$, 19-hydroxy-10(*R*),19-dihydrovitamin D_3.

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Table I: Failure of 19-Hydroxy-10(*R*),19-dihydrovitamin D_3 to Block Intestinal and Serum Calcium Responses to Vitamin D_3[a]

group	dose 1	time (h)	dose 2	^{45}Ca serosal/ ^{45}Ca mucosal	serum calcium (mg/100 mL)
1	ethanol	2.0	ethanol	2.0 ± 0.2 (12)	4.4 ± 0.1 (12)
2	ethanol	2.0	vitamin D_3 (50 ng)	4.3 ± 0.3 (11)[b]	5.4 ± 0.2 (12)[b]
3	×100 10*R*	1.0	vitamin D_3	4.2 ± 0.8 (5)[b]	5.8 ± 0.2 (5)[b]
4	×500 10*R*	1.0	vitamin D_3	4.7 ± 0.6[b]	5.4 ± 0.2[b]
5	×1000 10*R*	1.0	vitamin D_3	4.0 ± 0.2 (5)[b]	5.4 ± 0.2 (5)[b]
6	×100 10*R*	2.0	vitamin D_3	3.9 ± 0.3 (5)[b]	5.2 ± 0.2 (5)[b]
7	×100 10*R*	6.0	vitamin D_3	4.1 ± 0.2[b]	5.0 ± 0.1[c]
8	×100 10*R*	10.0	vitamin D_3	4.1 ± 0.5[b]	4.8 ± 0.1[d]
9	10*R* (26.2 μg)			2.3 ± 0.1	4.2 ± 0.2

[a] Data are given as the mean ± SEM of six rats per group unless otherwise noted by the number in parentheses. [b] Significantly different from control, $p < 0.001$. [c] Significantly different from control, $p < 0.01$. [d] Significantly different from control, $p < 0.05$.

& DeLuca (1969). For ^{45}Ca counting (Packard Tri-Carb Model 3375 liquid-scintillation counter) aliquots from the serosal and mucosal media were spotted on filter paper disks, dried, and placed in 20-mL counting vials containing 10 mL of toluene counting solution (2.0 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis(4-methyl-5-phenyl-2-oxazolyl)benzene per L of toluene).

Metabolism Studies. Six vitamin D deficient, low calcium rats (average weight 85.0 ± 10.0 g, mean ± SD) were dosed intrajugularly under light ether anethesia with 50 μL of ethanol containing a 500-fold (26.2 μg) excess of 19-OH-10(*S*),19-DHD_3 followed 2 h later by a 50-ng dose of vitamin D_3 which contained 1.0 μCi of [3α-^{3}H]vitamin D_3 in 50 μL of ethanol. A control group of six vitamin D deficient, low calcium rats (79.8 ± 8.3 g, mean ± SD) were dosed intrajugularly with 50 μL of ethanol followed 2 h later with a 50-ng dose of vitamin D_3 containing 1.0 μCi of [3α-^{3}H]vitamin D_3 in 50 μL of ethanol. The animals were sacrificed 20 h later, the blood was removed by cardiac puncture, and the entire liver was removed.

Serum samples were extracted according to Bligh & Dyer (1959) while liver homogenates (20%) were extracted according to Lund & DeLuca (1966). The serum extracts were chromatographed on a 2 × 62 cm column packed with Sephadex LH-20 in (65:35) $CHCl_3$/Skellysolve B. The columns were eluted with the same solvent, and 200 4.0-mL fractions were collected. The liver extracts were chromatographed as described above with only 65 4.0-mL fractions being collected. All fractions were evaporated and the residues redissolved in the toluene counting solution. Tritium content was determined by use of a Beckman LS-100C liquid-scintillation counter.

Metabolite levels are expressed as percent of the total administered dose present in the organ examined. These values are readily obtained for liver samples, since the entire organ was extracted. The total serum volume of a rat (in milliliters) was assumed to be 3% of the total body weight in grams (Ponchon & DeLuca, 1969). With this estimate and knowledge of the volume of serum extracted, calculation of the serum metabolite levels as percent of administered dose could be carried out.

Rat Liver Microsomal Incubations. This measurement was carried out according to the methods of Madhok & DeLuca (1979). The livers from vitamin D deficient 5-week-old male rats were removed and immediately placed in ice-cold buffer A (0.25 M sucrose, 10 mM EDTA, 15 mM Tris-HCl, and 1.15% KCl adjusted to pH 7.4 with 1.0 N NaOH). A 20% homogenate in buffer A was prepared from 15 g of the finely minced tissue by using a Potter–Elvehjem homogenizer fitted with a Teflon pestle. The homogenate was then subjected to centrifugation for 15 min at 500*g* (2500 rpm) (SS-34 head, Sorvall RC-5 centrifuge) to remove nuclear debris followed by a 15-min centrifugation at 10000*g* (9500 rpm) to remove the mitochondria. The microsomes were obtained by centrifuging the supernatant from the previous step for 65 min at 105000*g* (30 000 rpm), and the resulting pellet was resuspended in 10 mL of buffer A. The cytosolic and microsomal protein concentrations were determined by the method of Bradford (1976) and found to be ~22 mg/mL for both. Incubations were performed in 125-mL Erlenmeyer flasks by mixing 4.0 mL of the cytosolic fraction, 1.0 mL of the microsomal fraction, 2.5 mL of the buffer solution B [0.05 M $MgCl_2$, 0.1 M KCl, and 10 μL of 3.3 M $(NH_4)_2SO_4$ solution containing 3.25 units of glucose-6-phosphate dehydrogenase (Sigma Chemical Co., St. Louis, MO)], and 2.5 mL of a cofactor solution (0.1 M K_2HPO_4, 0.4 mM $NADP^+$, 160 mM nicotinamide, 20 mM ATP, and 0.4 mM glucose 6-phosphate). A 100 molar excess (10.5 μg) of 19-OH-10(*S*),19-DHD_3 was introduced in 10 μL of ethanol (control flasks received 10 μL of ethanol alone), and the flasks were flushed with oxygen for 30 s. After incubation at 37 °C and 120 oscillations/min for 10 min, 100 ng of [3α-^{3}H]vitamin D_3 (140000 cpm) was added to each flask and the incubation continued for 2.0 h. The reactions were terminated by the addition of 30 mL of a (2:1) MeOH/$CHCl_3$ solution. The remainder of the workup was the basic Bligh & Dyer technique (1959). After evaporation of the $CHCl_3$ phase and azeotropic (ethanol) removal of the residual water, the residue was applied to a 1 × 45 cm Lipidex-5000 column packed and eluted with hexanes/$CHCl_3$ (9:1). Three-milliliter fractions were collected for a total of 200 mL; vitamin D_3 was eluted in fractions 14–21 and 25-OH-D_3 in fractions 36–47.

Results

Both 19-OH-10(*R*),19-DHD_3 and 19-OH-10(*S*),19-DHD_3 were tested as antagonists of responses to vitamin D_3 and 25-OH-D_3 in the rat. Vitamin D deficient rats maintained on a low calcium diet received graded doses of either 19-OH-10(*R*),19-DHD_3 or 19-OH-10(*S*),19-DHD_3 intrajugularly in 50 μL of ethanol. The results for 19-OH-10(*R*),19-DHD_3 are presented in Table I. This compound was found to have no intrinsic vitamin D activity (group 9) and no antagonism of the expression of vitamin D activity even at a 1000-fold molar excess (52.3 μg) dose level (group 5). Table II summarizes the results obtained when 19-OH-10(*S*),19-DHD_3 was used as the predosed antagonist. This analogue, which possessed no intrinsic activity (group 18), showed significant antagonist activity at dose levels as low as a 25-fold (1.3 μg) excess over vitamin D_3 (group 6); however, only bone calcium mobilization was suppressed while the intestine retained the calcium transport response. This selective inhibition of bone calcium mobilization was noted over a wide range of inhibitor doses,

Table II: Intestinal and Bone Response to Vitamin D_3 and 25-OH-D_3 in Rats Given 19-Hydroxy-10(*S*),19-dihydrovitamin D_3[a]

group	dose 1	time (h)	dose 2	^{45}Ca serosal/ ^{45}Ca mucosal	serum calcium (mg/100 mL)
1	ethanol	2.0	ethanol	2.0 ± 0.1 (24)	4.5 ± 0.1 (24)
2	ethanol	2.0	vitamin D_3 (50 ng)	4.1 ± 0.2 (24)[b]	5.5 ± 0.1 (24)[b]
3	ethanol	2.0	25-OH-D_3 (25 ng)	4.5 ± 0.4[b]	5.5 ± 0.2[b]
4	×1 10*S*	2.0	vitamin D_3	3.2 ± 0.1[b]	5.7 ± 0.2[b]
5	×10 10*S*	2.0	vitamin D_3	3.9 ± 0.2[b]	5.4 ± 0.2[b]
6	×25 10*S*	2.0	vitamin D_3	3.9 ± 0.3[b]	4.6 ± 0.1
7	×50 10*S*	2.0	vitamin D_3	4.1 ± 0.3[b]	4.6 ± 0.1
8	×100 10*S*	2.0	vitamin D_3	3.8 ± 0.3 (12)[b]	4.6 ± 0.1 (12)
9	×100 10*S*	2.0	vitamin D_3 (150 ng)	4.2 ± 0.3[b]	5.1 ± 0.1[b]
10	×100 10*S*	2.0	vitamin D_3 (400 ng)	4.1 ± 0.3[b]	5.2 ± 0.1[b]
11	×1 10*S*	8.0	vitamin D_3	4.0 ± 0.4[b]	5.7 ± 0.1[b]
12	×10 10*S*	8.0	vitamin D_3	4.9 ± 0.7[b]	5.2 ± 0.1[b]
13	×100 10*S*	8.0	vitamin D_3	3.7 ± 0.2 (12)[b]	4.6 ± 0.1 (12)
14	×250 10*S*	8.0	vitamin D_3	4.2 ± 0.7[b]	4.6 ± 0.1
15	×500 10*S*	8.0	vitamin D_3	4.1 ± 0.4[b]	4.4 ± 0.2
16	×1000 10*S*	8.0	vitamin D_3	4.4 ± 0.4[b]	4.5 ± 0.1
17	×1000 10*S*	8.0	25-OH-D_3	4.4 ± 0.3[b]	5.3 ± 0.2[c]
18	×100 10*S*	0	vitamin D_3	3.2 ± 0.1[b]	4.5 ± 0.1
19	10 *S* (26.2 μg)			2.6 ± 0.2	4.6 ± 0.1

[a] Data are given as the mean ± SEM of six rats per group unless otherwise noted by the number in parentheses. [b] Significantly different from control, $p < 0.001$. [c] Significantly different from control, $p < 0.01$. [d] Significantly different from control, $p < 0.05$.

Table III: Effect of 19-Hydroxy-10(*S*),19-dihydrovitamin D_3 on Serum Vitamin D Metabolite Levels

	[^{3}H]vitamin D_3 dosed[a]		19-OH-10(*S*),19-DHD_3 and [^{3}H]-vitamin D_3 dosed[b]	
	ng/mL	% dose	ng/mL	% dose
vitamin D_3 ester	0.009	0.05	0.024	0.11
vitamin D_3	0.13	0.66	0.42	2.02
25-OH-D_3	0.69	3.5	0.27	1.30
peak V	0.37	1.9	0.092	0.44

[a] 6.5% of ^{3}H given was found in the plasma with 93% recovery following extraction and chromatography. [b] 4.9% of ^{3}H given was found in the plasma with 80% recovery following extraction and chromatography.

Table IV: Effect of 19-Hydroxy-10(*S*),19-dihydrovitamin D_3 on [^{3}H]Vitamin D_3 Liver Metabolite Levels

	[^{3}H]vitamin D_3 dosed[a] (% dose)	19-OH-10(*S*),-19-DHD_3 and [^{3}H]vitamin D_3 dosed[b] (% dose)
vitamin D_3 ester	0.5	0.72
vitamin D_3	0.52	3.31
25-OH-D_3	0.78	0.70

[a] 4.5% of ^{3}H given was found in the liver with 68% recovery following extraction and chromatography. [b] 7.9% of ^{3}H given was found in the liver with 76% recovery following extraction and chromatography.

Table V: Comparative [^{3}H]Vitamin D_3 Metabolite Levels in Plasma and Liver

	plasma[a]	liver[a]
total cpm	0.74	1.86
vitamin D_3 ester	2.58	1.44
vitamin D_3	3.23	6.37
25-OH-D_3	0.39	0.90
peak V	0.23	

[a] Data are expressed as the ratio of radioactivity found in the rats given 19-OH-10(*S*),19-DHD_3 plus [^{3}H]vitamin D_3 over those given only [^{3}H]vitamin D_3.

25 (1.3 μg)–1000 (52.3 μg)-fold molar excesses, for both 2 and 8 h predose time points (groups 6–8, 13–16). This inhibitory action was overcome when a physiologic dose (25 ng) of 25-OH-D_3 was administered (group 17) while threefold (150 ng) and eightfold (400 ng) increases in vitamin D_3 levels (groups 9, 10) result in partially elevated serum calcium levels.

The effect of 19-OH-10(*S*),19-DHD_3 on the functional metabolism of vitamin D_3 was studied by giving vitamin D deficient, low calcium rats either ethanol or the inhibitor (500-fold molar excess or 26.2 μg/rat) via intrajugular injection followed by a physiological dose of [3α-^{3}H]vitamin D_3 (50 ng) 2 h later by the same route. Twenty hours later the animals were sacrificed; serum and livers were extracted and chromatographed on Sephadex LH-20. Figure 1, a and b, shows the chromatographic profiles for the serum and liver samples, respectively, with the serum chromatograms being extended to show the "peak V" (dihydroxylated) metabolites. The inhibitor-dosed animals showed lower total radioactivity per milliliter of serum and elevated circulating levels of esterified vitamin D and free vitamin D_3. The levels of 25-OH-D_3 and "peak V" metabolites were significantly reduced by the 10*S* compound. Although the 25-OH-D_3 levels in liver were approximately equal in both inhibitor-dosed and control groups, the livers of inhibitor-dosed animals contained more total radioactivity which could be accounted for by the much higher levels of free vitamin D_3. Tables III and IV give quantitative summaries of these results, and Table V presents the data from the metabolic studies as comparative ratios.

The in vitro rat liver microsomal 25-hydroxylase experiments were performed in duplicate with a 100-fold molar excess of 19-OH-10(*S*),19-DHD_3 being added 10 min prior to a 100-ng dose of [3α-^{3}H]vitamin D_3. The control incubations gave 3.9% conversion of substrate to [^{3}H]25-OH-D_3 while pretreatment with the 10*S* inhibitor drastically reduced the amount of [^{3}H]25-OH-D_3 formed. Figure 2 shows the chromatographic profiles obtained from the in vitro experiments.

Discussion

Since the initial studies on the inhibitory properties of 19-OH-10(*S*),19-DHD_3 in the chick suggested that a 6- or 12-h predose of inhibitor was necessary for inhibitory activity (Norman et al., 1977), the predose time points in our study were varied in order to establish whether this time factor was critical in the rat. We found 19-OH-10(*S*),19-DHD_3 to be effective when codosed (group 19) with 50 ng of vitamin D_3

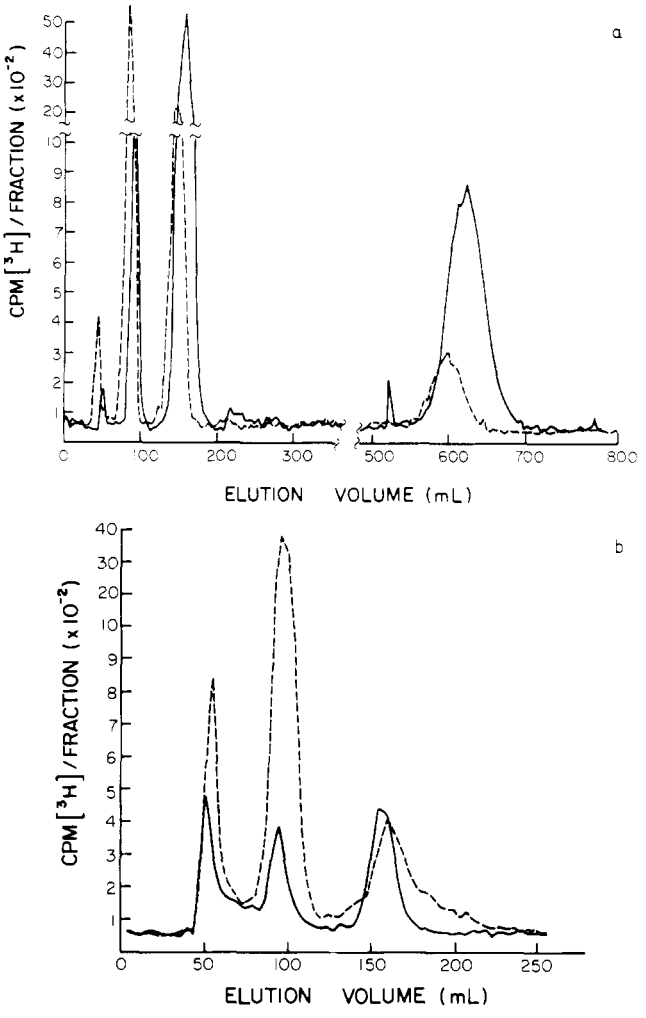

FIGURE 1: (a) Sephadex LH-20 chromatogram (2 × 62 cm column eluted in $CHCl_3$/Skellysolve B, 65:35) of serum extracts obtained from vitamin D deficient rats which were sacrificed 20 h after being dosed with either 50 ng of [^{3}H]vitamin D_3 (—) or 26.2 μg of 19-OH-10(*S*),19-DHD_3 2.0 h prior to 50 ng of [^{3}H]vitamin D_3 (- - -). (b) Sephadex LH-20 chromatogram (2 × 62 cm column eluted in $CHCl_3$/Skellysolve B, 65:35) of liver extracts obtained from vitamin D deficient rats which were sacrificed 20 h after being dosed with either 50 ng of [^{3}H]vitamin D_3 (—) or 26.2 μg of 19-OH-10-(*S*),19-DHD_3 2.0 h prior to 50 ng of ^{3}H-vitamin D_3 (- - -). In order of elution the compounds are (top) vitamin D_3 esters, vitamin D_3, 25-OH-D_3, and dihydroxyvitamin D metabolites and (bottom) vitamin D_3 esters, vitamin D_3, and 25-OH-D_3.

in addition to 2- and 8-h predoses.

The spectrum of activity for 19-OH-10(*S*),19-DHD_3 remained constant over a large range of doses, 1.3 μg (×25)–52.3 μg (×1000). The ability of the gut to transport calcium was not affected while bone calcium mobilization was inhibited at doses ≥1.3 μg (×25) of 19-OH-10(*S*),19-DHD_3. In contrast, the epimeric 19-OH-10(*R*),19-DHD_3 showed no inhibitory properties at a variety of dose levels and predose intervals (groups 1–8).

When 25-OH-D_3 was administered after a large dose of 19-OH-10(*S*),19-DHD_3, normal response was obtained in both assays. Similar results were obtained by Norman et al. (1977) in the chick, thus establishing the action of 19-OH-10-(*S*),19-DHD_3 at the 25-hydroxylation level, although the nature of this inhibition is unclear from the in vivo studies alone.

The effect of 19-OH-10(*S*),19-DHD_3 on the metabolism of [^{3}H]vitamin D_3 (Figure 1a) showed that circulating levels of 25-OH-D_3 are reduced to 40% of normal while peak V

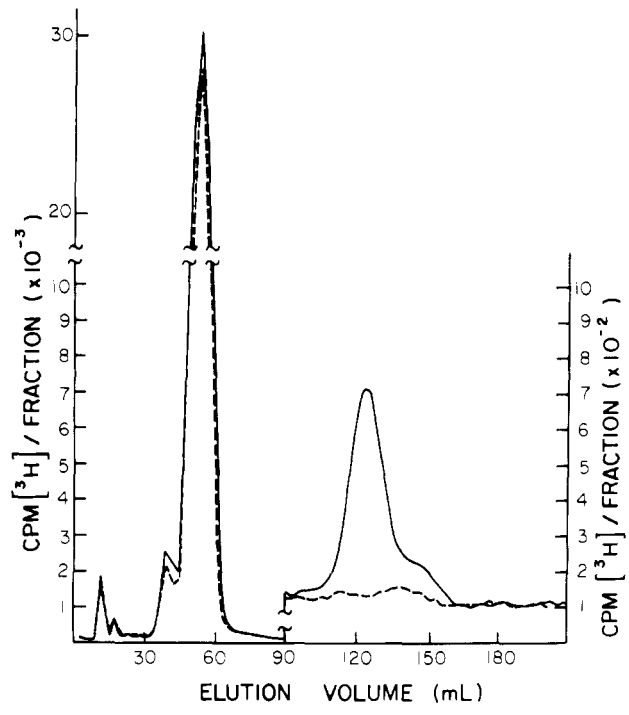

FIGURE 2: In vitro rat liver microsomal 25-hydroxylase activity determined by Lipidex-5000 chromatography (1 × 45 cm column eluted in hexanes/$CHCl_3$, 9:1). Incubations were conducted for 2.0 h with either 100 ng of [^{3}H]vitamin D_3 (—), yielding 3.9% conversion to 25-OH-D_3, or a 100-fold molar excess of 19-OH-10(*S*),19-DHD_3 added 10 min prior to 100 ng of [^{3}H]vitamin D_3 (- - -).

serum metabolites [dihydroxylated vitamin D compounds consisting mostly of 1α,25-$(OH)_2D_3$] are 20% of the control values. Serum levels of free vitamin D_3 and vitamin D_3 esters are much higher in the inhibitor-dosed animals, indicating that liver uptake of vitamin D_3 has become impaired at high endohepatic vitamin D_3 concentrations. Surprisingly the hepatic levels of 25-OH-D_3 in both groups are approximately equal (Figure 1b).

The apparent selective inhibition of bone calcium mobilization probably results from reduced circulating levels of 1α,25-$(OH)_2D_3$ which are not sufficient to activate the bone resorption mechanism but which are in high enough concentrations to stimulate the intestine. This conclusion is in accordance with the finding that the intestine is about 10 times more sensitive to 1α,25-$(OH)_2D_3$ than is the bone (Holick et al., 1975).

Although the metabolic studies provided an explanation for the observed partial expression of vitamin D activity, the actual mechanism of inhibition required further investigation. The reduction but not total elimination of 25-hydroxylase activity can be envisioned to occur via two possible inhibition pathways. 19-OH-10(*S*),19-DHD_3 might be blocking transport of 25-OH-D_3 from the liver, and 25-hydroxylase activity is being turned off by product inhibition. In light of the fact that hepatic 25-OH-D_3 levels are comparable in inhibitor-dosed and control animals, this may seem a reasonable explanation; however, additional evidence, i.e., high levels of vitamin D_3 partially overcome bone inhibition and extremely high doses of inhibitor do not affect gut transport, is not compatible with a transport block mechanism. An alternative explanation would cast 19-OH-10(*S*),19-DHD_3 in the role of a highly specific vitamin D_3-25-hydroxylase inhibitor capable of shutting down the major metabolic pathway to 25-OH-D_3 without affecting the less specific hepatic 25-hydroxylases (e.g., cholesterol 25-hydroxylase), which would metabolize vitamin

D_3 at high substrate concentrations (Bjorkhem & Holmberg, 1978). Because the microsomes are believed to be the major physiological site of 25-hydroxylation (Madhok & DeLuca, 1979), inhibition of the in vitro microsomal system would lend support for the latter mechanism. Inhibition was observed with a 100-fold excess of inhibitor (Figure 2) in such an assay. The unique inhibitory properties of 19-OH-10(*S*),19-DHD_3 therefore may result from its selective inhibition of a microsomal vitamin D_3-25-hydroxylase in conjunction with unaffected mitochondrial or exohepatic 25-hydroxylases. However, this will require a more direct investigation before it can be accepted.

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Changes in Membrane Potential and Membrane Fluidity in *Tetrahymena pyriformis* in Association with Chemoreception of Hydrophobic Stimuli: Fluorescence Studies†

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ABSTRACT: The fluorescence intensity of rhodamine 6G (Rh6G) and 1,6-diphenyl-1,3,5-hexatriene (DPH) in the presence of *Tetrahymena pyriformis* was measured to monitor changes in the membrane potential and in the gross structure of the surface membrane in response to chemical stimuli. So-called "odorants" for higher vertebrates, which are usually uncharged and hydrophobic compounds, were chosen as chemical stimuli for a model study of the olfactory response. The fluorescence intensity of Rh6G started to increase at the chemotactic thresholds of the stimuli, indicating that negative chemotaxis of *T. pyriformis* to the hydrophobic stimuli is induced by depolarization of the cell. The fluorescence intensity of DPH increased in close association with chemoreception of the hydrophobic stimuli. The increase in the fluorescence intensity was ascribed mainly to uptake of DPH, suggesting that gross structural changes of the surface membrane occur with the reception of hydrophobic stimuli. The membrane fluidity determined by fluorescence polarization of DPH increased in close association with the chemoreception of the hydrophobic stimuli. Inorganic salts such as NaCl, KCl, and $CaCl_2$ did not change the DPH fluorescence intensity or the fluorescence polarization, although these stimuli induced depolarization and negative chemotaxis in *T. pyriformis*.

Living organisms from unicellular organisms to higher vertebrates have an ability to recognize chemical stimuli in external environments. The response of unicellular organisms to chemical stimuli can be seen in chemotaxis. In higher vertebrates, chemical stimuli in the external environment are received at gustatory and olfactory cells. Recently much attention has been paid to the molecular mechanism of chemoreception in the sensory cells, but a detailed mechanism is still unknown. The difficulties encountered in exploring the mechanism come from the limitations of the techniques that can be applied; few techniques besides electrophysiological ones can be applied to intact sensory cells. Furthermore, in the case of olfactory cells, which are terminal swellings of olfactory nerves, intracellular recordings of electrical properties of the cells are extremely difficult because of the small size of the

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