5,6-Trans Isomers of Cholecalciferol and 25-Hydroxycholecalciferol. Substitutes for 1,25-Dihydroxycholecalciferol in Anephric Animals†

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ABSTRACT: Two analogs of vitamin D, 5,6-*trans*-cholecalciferol and 5,6-*trans*-25-hydroxycholecalciferol, have been synthesized and tested for their biological activity. The 5,6-*trans*-cholecaliciferol elicits an intestinal calcium-transport response as well as a bone calcium mobilization response in anephric rats while its 25-hydroxy derivative only stimulates intestinal calcium transport in these animals. By way of contrast, the 5,6-cis compounds (cholecalciferol and 25-hydroxycholecalciferol) are inactive in nephrectomized animals. An explanation for this unexpected biological activity of the trans isomers has been provided based on the fact that the 3-hydroxyl is geometrically located in the position normally occupied by the 1 hydroxyl of 1,25-dihydroxycholecalciferol, the probable metabolically active form of vitamin D_3 in the stimulation of intestinal calcium transport and calcium mobilization from bone.

The synthesis of various analogs of biologically active metabolites has provided compounds that possess varying degrees of biological activity and in some instances rather unusual biological properties. Windaus and coworkers (1934, 1935) were the first to attempt the synthesis of antirachitic compounds with the 5,6-*cis*-triene chromophore of vitamin D_2 but with differing side chain structures. They successfully synthesized an analog of ergosterol which had a cholesterol side chain and reported that one of its irradiation products possessed about the same antirachitic potency as vitamin D_2. Since these initial experiments, vitamin D has been chemically altered in various ways and the products tested for their biological activity. The dihydrotachysterols which were first synthesized and isolated by von Werder (1939) are one of the more useful analogs of vitamin D. Although they have very little antirachitic activity, this group of analogs displays preferential activity in elevating serum calcium concentration (Suda *et al.*, 1970a; Harrison *et al.*, 1968; Roborgh and DeMan, 1960). Various analogs of vitamin D have since been made but very little attention has been focused on them because they lack biological activity.

It was not until recently that it became clear that vitamin D must be multiply hydroxylated before it can function as a hormone for calcium homeostasis. Vitamin D is first hydroxylated in the liver at C-25 (Blunt *et al.*, 1968; Ponchon *et al.*, 1969; Horsting and DeLuca, 1969) and then in the kidney at C-1 (Fraser and Kodicek, 1970; Gray *et al.*, 1971) before it can stimulate either intestinal calcium transport (Boyle *et al.*, 1972) or bone calcium mobilization (Holick *et al.*, 1972). This concept led Suda *et al.* (1970a) to synthesize the 25-hydroxy derivative of dihydrotachysterol$_3$ and demonstrate that it is biologically more active than dihydrotachysterol$_3$. Similarly Hallick and DeLuca (1971) have shown that dihydrotachysterol$_3$ is hydroxylated at C-25 by rats and this hydroxylation occurs in the liver similar to the cholecalciferol 25-hydroxylase.

Because 1,25-dihydroxycholecalciferol (1,25-$(OH)_2D_3$)[1] now appears to be the metabolically active form of vitamin D in intestine and bone, it was of interest to synthesize the 5,6-trans isomers of vitamin D_3 and 25-hydroxycholecalciferol (25-OHD_3), because of their similarity in appearance especially in regard to the geometry of the hydroxyl function on C-1. Verloop *et al.* (1955) were the first to report the synthesis of the 5,6-trans isomer of vitamin D_2 (9,10-seco-5*E*,7*E*,22*E*-5,7,10(19,22)-ergostatetraene) by iodine catalyzed isomerization of vitamin D_2 under neutral conditions in a nonpolar solvent. Similarly, Inhoffen *et al.* (1957) studied this isomerization and reported the synthesis of 5,6-*trans*-vitamin D_3 (5,6-*trans*-cholecalciferol).

This report demonstrates that both 5,6-*trans*-cholecalciferol (5,6-trans-D_3) and 5,6-*trans*-25-hydroxycholecaliferol (5,6-trans-25-OHD_3) are biologically active analogs of vitamin D. They act in a similar fashion to 1,25-$(OH)_2D_3$ in that their biological response is sustained in anephric animals.

Experimental Procedure

Ultraviolet absorption spectra were determined in diethyl ether with a Beckman DB-G spectrophotometer, while mass spectrometric determinations were carried out with an A.E.I. MS-9 mass spectrometer, using direct probe inlet at temperatures of 110–130° above ambient. Gas–liquid partition chromatography (glc) was performed in an F & M Model 402 gas chromatograph using a 4 ft × 0.25 in. glass column packed with 3% SE-30 on Gas-Chrom Z, 100–120 mesh (Applied Science Labs., Inc., State College, Pa.). The column temperature was 250° and an outlet flow rate of 80 cm^3/min was maintained. All solvents used were of reagent grade.

Radioactive determinations were carried out with a Packard Tri-Carb Model 3375 liquid scintillation counter equipped with an automatic external standardization system. Samples were counted in 10 ml of a toluene-counting solution consisting of 2 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene per l. of toluene.

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[1] Abbreviations used are: 1,25-$(OH)_2D_3$, 1,25-dihydroxycholecalciferol; 25-OHD_3, 25-hydroxycholecalciferol; 5,6-trans-D_3,5,6-*trans*-cholecalciferol; 5,6-trans-25-OHD_3, 5,6-*trans*-25-hydroxycholecalciferol.

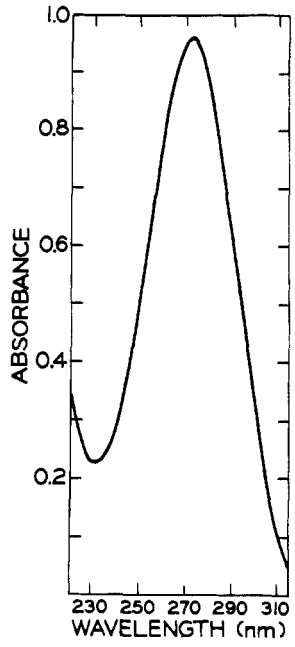

FIGURE 1: Ultraviolet absorption spectrum for 5,6-trans-D_3.

Synthesis of 5,6-Trans-D_3. Vitamin D_3 (0.5 g) was dissolved in 10 ml of Skellysolve B (predominantly *n*-hexane redistilled at 67–69°). To this was added 0.1 ml of a solution of iodine (1 mg/ml) and the isomerization was monitored by glc. After 1 hr the glc tracing showed a 9:11 ratio of 5,6-trans-D_3:vitamin D_3. After removal of I_2 by means of sodium thiosulfate, washing and drying over anhydrous Na_2SO_4, the product was acetylated in 5 ml of acetic anhydride/0.1 ml of pyridine at 50° for 6 hr. The reaction mixture was extracted with diethyl ether (Et_2O) and H_2O (pH −4). The ether phase was collected and the aqueous phase was extracted twice with Et_2O. The ether phases were combined and dried by flash evaporation. The product was dissolved in 0.5 ml of Skellysolve B and applied to a 5 g of silver nitrate impregnated silicic acid column (1.1 × 10 cm) containing 1 g of Celite as filler. The column was developed with 50 ml of 1:20 Et_2O–Skellysolve B and then with an additional 300 ml of 1:10 Et_2O–Skellysolve B; 10-ml fractions were collected. Vitamin D_3 was found in fractions 5–7 and the 5,6-trans isomer was located in fractions 12–30. This chromatographic procedure was repeated once more to insure that there was no vitamin D_3 with the 5,6-trans isomer. The 5,6-trans isomer (200 mg) was collected after the second chromatography, the solvent evaporated with a stream of N_2, and the isomer dissolved in 10 ml of Et_2O. $LiAlH_4$ (50 mg) was added and the reaction continued for 1 hr at 25°. The reaction was extracted with H_2O (pH −4)

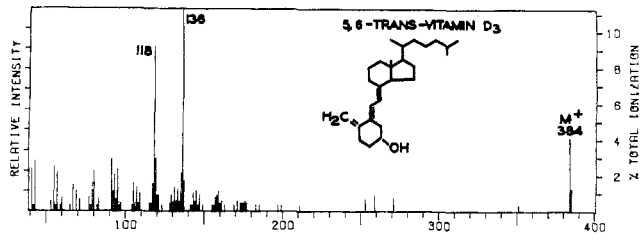

FIGURE 2: Mass spectrum of 5,6-trans-D_3.

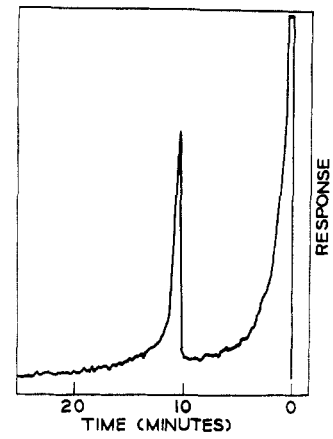

FIGURE 3: Gas–liquid partition chromatography of 5,6-trans-D_3.

and the aqueous phase was reextracted with Et_2O. The Et_2O phases were combined, dried under N_2, and redissolved in 0.5 ml of Skellysolve B. The sample was applied to a 15-g multibore silicic acid column (dimensions 1.5 × 15, 1.0 × 15, 0.8 × 15, and 0.6 × 15 cm) that had been packed in the same solvent. The column was developed with a hyperbolic gradient produced with a holding chamber containing 300 ml of 3:7 Et_2O–Skellysolve B connected to a mixing chamber containing 230 ml of Skellysolve B. The column was run under 4–5 psi and 5.1-ml fractions were collected. Fractions 26–30 containing 5,6-trans-D_3 were combined, dried under N_2, and dissolved in Et_2O for glc, mass spectral, and ultraviolet absorption analysis.

The ultraviolet spectrum for 5,6-trans-D_3 (Figure 1) showed the characteristic λ_{max} 273.5 nm and a λ_{min} 232 nm (Verloop *et al.*, 1955) for the 5,6-*trans*-triene system. The mass spectrum of the trans isomer (Figure 2) showed a molecular ion peak at *m/e* 384, similar to vitamin D_3 and fragments at *m/e* 271 and 253 (271 − H_2O) characteristic for the loss of the side chain and peaks at *m/e* 136 and 118 (136 − H_2O) characteristic for the A ring plus C_6 and C_7. The mass spectrum for the 5,6-trans isomer was almost identical to vitamin D_3 except for fragment *m/e* 259. In contrast to vitamin D_3, the 5,6-trans isomer showed only one peak on the glc column (Figure 3) (Verloop *et al.*, 1959). The glc trace also demonstrated the relative purity of the 5,6-trans-D_3.

Synthesis of 5,6-Trans-25-OHD_3. The 5,6-trans isomerization of 25-OHD_3 was conducted in a similar fashion as described above for vitamin D_3. 25-OHD_3(10 mg) was dissolved in 10 ml of 1:9 Et_2O–Skellysolve B and 50 μl of a 0.1 mg of I_2/1 ml of Skellysolve B was added. After 1 hr at 25° the reaction was treated with sodium thiosulfate, washed, the ether phases combined, and dried under N_2. [26,27-^{3}H]25-OHD_3 (0.3 μg; specific activity 9.2 × 10^6 dpm/μg) (Suda *et al.*, 1971) was added to the sample and then applied in 0.5 ml of 3:7 Et_2O–Skellysolve B to a 15-g multibore silicic acid column as described above. The holding chamber contained 500 ml of 7:3 Et_2O–Skellysolve B and the mixing chamber contained 230 ml of 3:7 Et_2O–Skellysolve B; 6.1-ml fractions were collected. The 5,6-trans-25-OHD_3 in fractions 50–57 was chromatographed three times more under identical conditions such that there was less than 0.1% of 25-OHD_3 in the 5,6-trans-25-OHD_3 based on the radioactivity due to [26,27-^{3}H]25-OHD_3.

The ultraviolet spectrum for the 5,6-trans-25-OHD_3 was identical with that of 5,6-trans-D_3 (Figure 1) and the mass

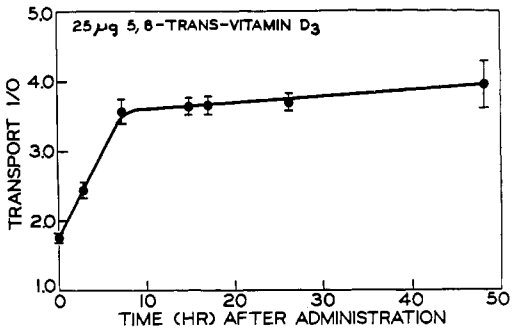

FIGURE 4: Intestinal calcium transport response of vitamin D deficient rats on a low calcium diet to a 25-μg dose of 5,6-trans-D_3. The verticle bars represent the standard error of the mean for six animals.

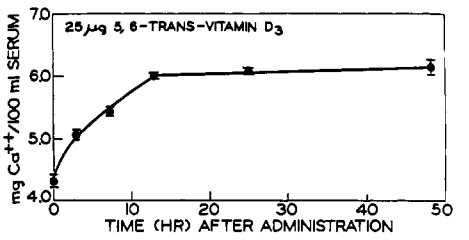

FIGURE 5: Bone calcium mobilization response of vitamin D deficient rats on a low calcium diet to a 25-μg dose of 5,6-trans-D_3. The verticle bars represent the standard error of the mean for six animals.

spectrum of the analog showed a molecular ion peak at *m/e* 400 and fragments similar to 25-OHD_3.

Synthesis of 24-Nor-25-OHD_3. The synthesis of 24-nor-25-OHD_3 was conducted according to the procedure of Blunt and DeLuca (1969) for 25-OHD_3, starting with 20 mg of 24-nor-25-hydroxycholesterol (kindly supplied by Dr. John Babcock of the Upjohn Co., Kalamazoo, Mich.). The final product was verified based on its ultraviolet absorption spectrum, mass spectrum and glc trace.

The product showed a λ_{max} of 265 nm and λ_{min} 228 nm characteristic for the 5,6-*cis*-triene system of vitamin D (Blunt *et al.*, 1968) and a mass spectrum similar to 25-OHD_3 except that the molecular ion peak at *m/e* 386 was 14 mass units less. The glc trace of the product showed the characteristic pyro and isopyrovitamin D peaks (Ziffer *et al.*, 1960).

Synthesis of 24-Nor-5,6-trans-25-OHD_3. 24-Nor-25-OHD_3 (1 mg) was isomerized to the 5,6-trans isomer and purified according to the procedure described above for 5,6-trans-25-OHD_3. Ultraviolet absorption spectrum, mass spectrum, and a glc tracing showed the product to be the 5,6-trans isomer of 24-nor-25-OHD_3.

Biological Assays

Animals. Weanling male rats (Holtzman Co., Madison, Wis.) were housed in overhanging wire cages. They were fed *ad libitum* a vitamin D deficient low calcium diet (Suda *et al.*, 1970b) for three weeks. At the end of the third week the rats were approximately 80–90 g and had an average serum calcium cohcentration of 4.5 mg/100 ml of serum.

Intestinal Calcium Transport Assay. Groups of five to seven rats which were either bilaterally nephrectomized, sham operated or intact, received either 5,6-trans-D_3, 5,6-trans-25-OHD_3, 24-nor-5,6-trans-25-OHD_3, 25-OHD_3, or 24-nor-25-OHD_3 intrajugularly in 0.05 ml of 95% ethanol. At the desired time after dosing, the animals were decapitated and the blood and duodena were collected. The duodena were prepared according to the procedure of Martin and DeLuca (1969) for measuring intestinal calcium transport activity by the everted gut sac technique. Both the inside and outside (50 μl) were spotted on filter paper disks, dried, and placed in 20-ml counting vials containing 10 ml of scintillation counting solution.

Bone Calcium Mobilization. The blood from the rats was centrifuged and 0.1 ml of serum was mixed with 1.9 ml of a 0.1% $LaCl_3$ solution. Serum calcium concentration was determined with an atomic absorption spectrometer.

In order to obtain bone calcium mobilization data from anephric rats, weanling male rats were fed the adequate calcium and phosphorus vitamin D deficient diet for 2 weeks (Guroff *et al.*, 1963) in order to build up calcium stores in the bone and then were fed a low calcium (0.02%) vitamin D deficient diet for another 10 days (Suda *et al.*, 1970b). Groups of rats were bilaterally nephrectomized as described above and immediately after surgery were injected with the appropriate compound. Twenty-four hours later the blood was collected and the serum calcium was determined.

Results

The results shown in Figures 4 and 5 demonstrate that the 5,6-trans analog of vitamin D_3 is capable of stimulating both intestinal calcium transport and bone calcium mobilization. This analog elicits an intestinal calcium transport response as early as 3 hr after its administration and shows a maximum response at 6 hr. Similarly this compound demonstrates a small but significant bone calcium mobilization response after 3 hr and a maximum response at 12 hr. Table I shows that as little as 2.5 μg of this compound can initiate both of these responses. The most revealing observation about the 5,6-trans-D_3 analog is that it will produce both a bone calcium mobilization and intestinal calcium transport response in anephric rats similar to 1,25-$(OH)_2D_3$ (Boyle *et al.*, 1972; Holick *et al.*, 1972) while 25-OHD_3 cannot (Table II).

Although it might be expected that the 25-hydroxy deriva-

TABLE I: Response of Intestinal Calcium Transport and Bone Calcium Mobilization Systems to Various Doses of 5,6-Trans-D_3.[a]

Dose	^{45}Ca Serosal/ ^{45}Ca Musocal	Serum Calcium (mg %)
50 μl of 95% EtOH	1.9 ± 0.1[b] (6)	4.2 ± 0.1
0.25 μg of 5,6-trans-D_3	2.1 ± 0.1 (6)	4.4 ± 0.1
2.5 μg of 5,6-trans-D_3	3.6 ± 0.2 (6)	5.3 ± 0.1
5.0 μg of 5,6-trans-D_3	3.6 ± 0.1 (6)	6.0 ± 0.1
10.0 μg of 5,6-trans-D_3	3.4 ± 0.1 (6)	5.9 ± 0.1

[a] Rats were fed a low calcium vitamin D deficient diet for three weeks at which time they received the 5,6-trans-D_3 intravenously in 0.05 ml of 95% ethanol. Twenty-four hours later the animals were sacrificed for serum calcium determination and intestinal calcium-transport measurement. [b] Plus or minus the standard error of the mean. The numbers in parentheses represent the number of rats in each group.

TABLE II: Bone Calcium Mobilization Response to Various Analogs of Vitamin D_3 in Anephric Rats.[a]

Dose	Serum Calcium (mg %)
50 μl of 95% EtOH	4.3 ± 0.1[b] (6)
25 μg of 5,6-trans-25-OHD_3	4.9 ± 0.1 (6)
25 μg of 5,6-trans-D_3	6.6 ± 0.2 (6)
0.25 μg of 1,25-$(OH)_2D_3$	6.1 ± 0.1 (6)

[a] Rats were fed the 0.3% phosphorus and 0.47% calcium diet for 2 weeks and then fed the low calcium vitamin D deficient diet for 10 days. All animals were bilaterally nephrectomized and then given the indicated dose intravenously. Twenty-four hours later they were sacrificed for the determination of serum calcium concentration. [b] Plus or minus the standard error of the mean. The numbers in parentheses represent the number of rats in each group.

TABLE III: Intestinal Calcium Transport and Bone Calcium Mobilization Response to Various Analogs of Vitamin D_3 in Normal and Anephric Rats.[a]

Dose	Condn of Animal	^{45}Ca Serosal/^{45}Ca Mucosal	Serum Calcium (mg %)
50 μl of 95% EtOH	Normal	1.8 ± 0.2[b] (5)	4.2 ± 0.1
25 μg of 5,6-trans-25-OHD_3	Normal	4.4 ± 0.3 (5)	
25 μg of 25-OHD_3	Normal	4.5 ± 0.8 (5)	6.4 ± 0.1
25 μg of 24-nor-25-OHD_3	Normal	2.4 ± 0.2 (6)	6.1 ± 0.1
50 μl of 95% EtOH	Anephric	1.5 ± 0.1 (3)	
25 μg of 5,6-trans-25-OHD_3	Anephric	3.3 ± 0.3 (6)	
25 μg of 25-OHD_3	Anephric	1.9 ± 0.2 (5)	
25 μg of 24-nor-25-OHD_3	Anephric	1.5 ± 0.2 (6)	
25 μg of 5,6-trans-D_3	Anephric	3.3 ± 0.3 (6)	
20 μg of 24-nor-5,6-trans-25-OHD_3	Anephric	2.0 ± 0.1 (6)	

[a] Rats were treated exactly as described in Table I except for the doses indicated above. [b] Plus or minus the standard error of the mean. The numbers in parentheses represent the number of rats in each group.

tive of this analog would be more active than 5,6-trans-D_3, similar to the results with dihydrotachysterol$_3$ and 25-hydroxydihydrotachysterol$_3$, it was a surprise to find that this is not the case. It has a marked effect on inducing intestinal calcium transport in both normal and anephric rats (Table III) but has little if any ability to stimulate mobilization of calcium from the bone (Table II).

In light of the striking biological activity of the 5,6-trans isomers, it was of interest to explore the potential biological activity of the analogs of 25-OHD_3 and 5,6-trans-25-OHD_3 which had one less carbon in the side chain (Figure 6). Table III illustrates that 25 μg of 24-nor-25-OHD_3 is about 50% as active as 25μg of 25-OHD_3 in stimulating intestinal calcium transport whereas the bone calcium mobilization response has only decreased slightly.

Discussion

The 5,6-trans analogs of vitamin D_3 and 25-OHD_3 have been synthesized and their biological activity tested. The results in this report clearly demonstrate the efficacy of the 5,6-trans-D_3 in stimulating both intestinal calcium transport and bone calcium mobilization. The 5,6-trans-25-OHD_3, however, demonstrates the unusual property of stimulating intestinal calcium transport while having little if any ability in stimulating calcium mobilization from the bone.

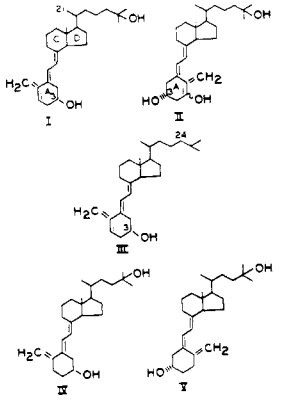

FIGURE 6: Structures of (I) 5,6-trans-25-OHD_3, (II) 1,25-$(OH)_2D_3$, (III) 5,6-trans-D_3, (IV) 24-nor-5,6-trans-25-OHD_3, and (V) 24-nor-25-OHD_3.

Of great importance is that both of these analogs are biologically active in anephric rats. Recently it has been shown by Fraser and Kodicek (1970) and later by Gray *et al.* (1971) that the kidney is the sole site for the C-1 hydroxylation of 25-OHD_3 while Boyle *et al.* (1972) and Holick *et al.* (1972) have demonstrated that the kidney tissue is essential for 25-OHD_3 but not 1,25-$(OH)_2D_3$ to function in inducing intestinal calcium transport and bone calcium mobilization. These results, therefore, demonstrate the strict requirement for a hydroxyl function at C-1 before vitamin D is biologically active in these tissues.

The 5,6-trans isomers of both vitamin D_3 and 25-OHD_3 appear to meet this essential requirement. Compared to the 5,6-cis chromophore of the D vitamins, the 5,6-trans isomer has its A ring rotated 180° thereby fortuitously placing the 3β-hydroxyl function in a similar geometric position as the 1-hydroxyl of 1,25-$(OH)_2D_3$ (Figure 6). It is probably for this reason that both 5,6-trans-D_3 and 5,6-trans-25-OHD_3 are biologically active in anephric animals similar to 1,25-$(OH)_2D_3$ and 25-hydroxydihydrotachysterol$_3$ (Hallick and DeLuca, 1972).

The biological activity of the 5,6-trans analogs and the 24-nor analogs of 25-OHD_3 and 5,6-trans-25-OHD_3 may shed some light on what are the essential parts of the vitamin D structure necessary for its various biological functions. Since the 5,6-trans-D_3 and 5,6-trans-25-OHD_3 are both effective in stimulating intestinal calcium transport, little can be said except that there appears to be little if any need for the hydroxyl function in the geometrical position at C-3 of the D vitamins after a hydroxyl is in the approximate C-1 position. The virtual lack of a bone calcium mobilization response

with 5,6-trans-25-OHD_3 while the 5,6-trans-D_3 elicited a significant response suggests that the hydroxyl function at C-25 is not a necessary criterion for biological activity in the bone and that the 5,6-trans isomer does not have to be hydroxylated at C-25 before it is active. The major function for the 25-hydroxylation may be to facilitate its transport to the kidney and for the eventual hydroxylation at C-1. Of special interest is that 25 μg of 24-nor-25-OHD_3 has about the same activity as 25 μg of 25-OHD_3 in stimulating a bone response while it is about 50% as effective in inducing intestinal calcium transport, demonstrating once again that there can be some variability in the side chain without destroying completely its biological activity.

In order to dispell any fear that large quantities of vitamin D-like compounds would nonspecifically induce intestinal calcium absorption and bone resorption it was shown that the biological activity induced by 24-nor-25-OHD_3 was completely blocked in anephric rats similar to 25-OHD_3. It was only after the A ring was rotated 180° by a cis–trans isomerization that the 24-nor-5,6-trans-25-OHD_3 showed a significant effect on intestinal calcium transport in anephric animals.

The role of the kidney as the sole site for the biosynthesis of a polar metabolite of vitamin D_3, recently identified as 1,25-$(OH)_2D_3$ (Holick *et al.*, 1971a, 1971b; Lawson *et al.*, 1971), has provided a new insight in vitamin D mediated calcium metabolism. This major advance provides a better understanding as to why patients who are on regular hemodialysis or with impaired renal function cannot adequately control their calcium metabolism. Hallick and DeLuca (1972) have suggested the 25-hydroxydihydrotachysterol$_3$ or dihydrotachysterol$_3$ may substitute for 1,25-$(OH)_2D_3$ in these situations since 25-hydroxydihydrotachysterol$_3$ can induce intestinal calcium transport in anephric rats.

The results in this report offer two new analogs of vitamin D which are inexpensive to prepare and can substitute for 1,25-$(OH)_2D_3$ in anephric rats. 5,6-Trans-D_3 can stimulate both intestinal calcium transport and bone calcium mobilization in anephric rats similar to 1,25-$(OH)_2D_3$. The unusual ability of the 5,6-trans-25-OHD_3 to induce intestinal calcium transport while having little if any effect on mobilization of calcium from the bone in anephric animals makes this analog extremely interesting as a possible drug in the treatment of calcium abnormalities associated with chronic renal failure. Certainly it would be desirable to stimulate calcium absorption in these patients without labilizing further existing bone stores of calcium. In any case, the potential of these analogs in the treatment of vitamin D resistant bone disease is clearly indicated by the present study.

Acknowledgment

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