Metabolism and Biological Activity of 25-Fluorocholecalciferol, 24-Dehydrocholecalciferol and 25-Dehydrocholecalciferol in the Rat

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Three side-chain analogues of cholecalciferol (vitamin D₃) modified at C-25, namely 25-fluorocholecalciferol, 24-dehydrocholecalciferol and 25-dehydrocholecalciferol, conceived as potential inhibitors of the cholecalciferol 25-hydroxylase have been prepared and tested in the rat. These compounds markedly diminish conversion *in vivo* of cholecalciferol into 25-hydroxycholecalciferol, but are not antagonists of vitamin D action, because they themselves possess significant biological activity *in vivo*. Each compound is capable of stimulating the intestinal transport of calcium and the mobilization of calcium from bone in vitamin D-deficient rats. Biological responses equivalent to those generated by a physiological dose of cholecalciferol (0.05 µg) are produced, however, only when the analogues are administered at high doses (5-50 µg). The biological activity of 24-dehydrocholecalciferol and 25-dehydrocholecalciferol is shown to result from conversion, *in vivo*, to the natural hormone, 1α,25-dihydroxycholecalciferol, whereas 25-fluorocholecalciferol is metabolically activated in the rat by hydroxylation to 1α-hydroxy-25-fluorocholecalciferol. This latter conversion is the first reported example of the 1α-hydroxylation of a vitamin D compound lacking the 25-hydroxy group.

Two of the better understood physiological responses of animals to cholecalciferol (vitamin D₃), namely the stimulation of intestinal absorption of calcium and increased mobilization of calcium from bone, are known to result from the direct action of the hormone 1α,25(OH)₂D₃, the product of two successive hydroxylations of vitamin D catalysed by mixed-function oxygenases, of which one (at C-25) occurs primarily in the liver, the other (at C-1) exclusively in the kidney (DeLuca & Schnoes, 1976). For C-1 hydroxylation, the key step of this metabolic activation of vitamin D, 25(OH)D₃ serves as the normal precursor *in vivo*, and, since cholecalciferol itself is not a substrate for the kidney 1-hydroxylase (Gray *et al.*, 1972), it appears that the side-chain C-25 hydroxy group is a necessary prerequisite for subsequent introduction of the ring-A hydroxy-group function. This information suggests that inhibitors of 25-hydroxylation that possess no intrinsic biological activity should be effective antagonists of vitamin D activation, and consequently, of vitamin D action. As

Abbreviations used: 1α,25(OH)₂D₃, 1α,25-dihydroxycholecalciferol; 25(OH)D₃, 25-hydroxycholecalciferol; 25(N)D₃, 25-azacholecalciferol; 25(F)D₃, 25-fluorocholecalciferol; Δ²⁴-D₃, 24-dehydrocholecalciferol; Δ²⁵-D₃, 25-dehydrocholecalciferol; h.p.l.c., high-pressure liquid chromatography.

a test of this concept, we prepared four side-chain-modified vitamin D compounds, namely 25(N)D₃, 25(F)D₃, Δ²⁴-D₃ and Δ²⁵-D₃, conceived as substrate analogues of the cholecalciferol 25-hydroxylase. These synthetic analogues (Fig. 1) feature a blocked C-25 position, but otherwise normal vitamin D carbon skeleton, a structural combination designed to prevent direct hydroxylation by the cholecalciferol 25-hydroxylase while preserving high affinity for the active site of the enzyme. Failure of these analogues to be 25-hydroxylated should in turn prevent subsequent C-1 hydroxylation and thus assure the absence of a vitamin D-like biological response to the compounds. We have shown previously that 25(N)D₃, a biologically inactive analogue, does indeed inhibit the 25-hydroxylation of cholecalciferol *in vivo*, and does antagonize the physiological action of cholecalciferol (Onisko *et al.*, 1979a). We report here that the other three analogues, 25(F)D₃, Δ²⁴-D₃ and Δ²⁵-D₃, although capable of inhibiting the cholecalciferol 25-hydroxylase, are not vitamin D antagonists, because they possess intrinsic biological activity. In the case of the two dehydro compounds, this activity results from their conversion *in vivo*, in low yield, to 1α,25(OH)₂D₃, whereas the biological responses elicited by 25(F)D₃ are a consequence of its metabolism to 1α-hydroxy-25-fluorocholecalciferol.

Fig. 1. Structures of cholecalciferol (vitamin D_3 ; D_3), two of its natural metabolites [$25(OH)D_3$ and $1\alpha,25(OH)_2D_3$], four side-chain-modified analogues [$25(N)D_3$, $25(F)D_3$, $\Delta^{24}-D_3$ and $\Delta^{25}-D_3$] and of $1\alpha(OH)25(F)D_3$, a metabolite of $25(F)D_3$

Experimental

General methods

Mass spectra were obtained on an AEI model MS-902 mass spectrometer at 70 eV, with a direct probe for introduction of samples (source temperature 110–130°C above ambient); high-resolution mass spectra were measured on the same instrument coupled to an AEI model DS-50 data system and by using perfluorokerosene as internal mass standard. U.v.-absorption spectra were recorded on a Beckman model 25 instrument. N.m.r. spectra were taken on a Bruker 270 MHz spectrometer by using $[^2H]$ chloroform as solvent and tetramethylsilane as internal standard. G.l.c. was carried out on a Packard model 417 chromatograph equipped with a glass column (2 mm $\times$ 2 m) packed with 3% OV-101 on Chromosorb 30 (100–200 mesh), operated isothermally at 260°C with N_2 as carrier gas at a flow rate of 20 ml/min. Analytical h.p.l.c. was performed on a Dupont model

830 instrument equipped with a Waters model U6K injector and a 254 nm u.v. detector. For $AgNO_3$ -impregnated column chromatography, 100 g of silica gel (Davison Chemical grade 923, 100–200 mesh) was added to a solution of 50 g of $AgNO_3$ in water (200 ml), heated at 100°C for 30 min, cooled, filtered, and then activated for 12 h at 115°C. Sephadex LH-20 (Pharmacia) was swelled in column solvent overnight before column packing. For analytical silica-gel t.l.c., air-dried silica-gel G plates (5 cm $\times$ 20 cm, 0.25 mm thick) were prepared from a de-gassed slurry of silica-gel G (30 g) in water (65 ml). For preparative silica-gel t.l.c., air-dried plates (20 cm $\times$ 20 cm, 0.75 mm thick) were made from a de-gassed slurry of silica-gel H (45 g) and silica-gel PF-254 (45 g) in water (194 ml). Commercial Skellysolve B was distilled and the fraction boiling at 67–69°C was used.

Compounds

Cholecalciferol was purchased from Aldrich

Chemical Co. (Milwaukee, WI, U.S.A.); 25(F)D₃ was synthesized as described previously (Onisko *et al.*, 1977; Onisko *et al.*, 1979b). 1 α (OH)25(F)D₃ and 24R(OH)25(F)D₃ were prepared by Dr. J. Napoli (see Napoli *et al.*, 1978).

Preparation of 24-dehydrocholecalciferol and 25-dehydrocholecalciferol

To a solution of 25(OH)D₃ 3-acetate (53mg) in 3.5ml of pyridine, cooled to ice-bath temperature, phosphorus oxychloride (350mg) was added dropwise with stirring. The ice bath was removed, and stirring was continued at ambient temperature for 1.0h. The reaction was stopped by cooling to 0°C, adding 5ml of water dropwise and diluting with 30ml of benzene. The organic layer was separated, washed with 5% (v/v) HCl (4 $\times$ 10ml), 4% NaHCO₃ and saturated NaCl (10ml). After drying the material over Na₂SO₄, the solvent was evaporated and the residue was applied to a 70g (1.2cm $\times$ 75cm) column of silica gel containing 10% (w/w) AgNO₃. The column was eluted with a hyperbolic gradient of ethyl acetate in Skellysolve B [the bottom reservoir contained 230ml of 5% (v/v) ethyl acetate in Skellysolve B; the top reservoir contained 230ml of 10% ethyl acetate in Skellysolve B; 6.5ml-fractions were collected]. Fractions 25–34 were pooled and concentrated, and the residue was further purified by preparative t.l.c. (silica gel, 5% ethyl acetate in Skellysolve B). The major band was eluted with ethyl acetate. Solvent removal gave 19.0mg (37%) of 24-dehydrocholecalciferol 3-acetate as an oil: u.v. (ethanol) $\lambda_{\max}$. 264nm; n.m.r. (270MHz) δ (p.p.m.) ([²H]chloroform) 6.22 and 6.03 (AB, *J* 11Hz, 2H, C-6,7), 5.09 (t, *J* 7Hz, 1H, C-24), 5.06 (m, 1H, C-19), 4.94 (t of t, *J* 8 and 4Hz, 1H, C-3), 4.83 (d, *J* 2.6Hz, 1H, C-19), 2.04 (s, 3H, acetate), 1.68 (s, 3H, C-26), 1.60 (s, 3H, C-27), 0.94 (d, *J* 6.3Hz, 3H, C-21), 0.54 (s, 3H, C-18); mass spectra: *m/e* (intensity relative to base peak) 424 (M⁺, 26), 364 (65), 349 (6), 253 (12), 158 (37), 118 (100), 69 (44), 55 (28); homogeneous on t.l.c. (*R_F* = 0.55; 5% ethyl acetate in Skellysolve B). Similarly, pooling of fractions 39–53 from the silica-gel/AgNO₃ column and further purification by preparative t.l.c. (silica gel; 5% ethyl acetate in Skellysolve B) gave 5.6mg (11%) of 25-dehydrocholecalciferol 3-acetate as an oil: u.v. (ethanol) $\lambda_{\max}$. 265nm; n.m.r. (270MHz) δ (p.p.m.) ([²H]chloroform) 6.22 and 6.03 (AB, *J* 11Hz, 2H, C-6,7), 5.06 (m, 1H, C-19), 4.94 (t of t, *J* 7.8 and 3.9Hz, 1H, C-3), 4.84 (d, *J* 2Hz, 1H, C-19), 4.68 (s, 1H, C-26), 4.66 (s, 1H, C-26), 2.04 (s, 3H, acetate), 1.71 (s, 3H, C-27), 0.93 (d, *J* 6.2Hz, 3H, C-21), 0.55 (s, 3H, C-18); *m/e* (relative intensity) 424 (M⁺, 25), 364 (70), 349 (7), 253 (16), 158 (35), 118 (100), 69 (22), 55 (37); homogeneous on t.l.c. (*R_F* = 0.55; 5% ethyl acetate in Skellysolve B).

24-Dehydrocholecalciferol. 24-dehydrocholecalciferol

3-acetate (16.0mg) was dissolved in 3.8ml of methanolic 0.1M-KOH and kept at 25°C under N₂ for 1h. After adding 10ml of water, the reaction mixture was extracted with chloroform (3 $\times$ 10ml). The combined chloroform extracts were washed with 5% HCl (10ml), and saturated NaCl solution (10ml), then dried (over anhydrous Na₂SO₄) and concentrated by flash evaporation. The resulting residue was purified by preparative t.l.c. on a silica-gel plate developed with 20% ethyl acetate in Skellysolve B. The major band was eluted from the adsorbent with ethyl acetate. This gave 11.4mg (79%) of 24-dehydrocholecalciferol as an oil: u.v. (ethanol) $\lambda_{\max}$. 265nm; n.m.r. (270MHz) δ (p.p.m.) ([²H]chloroform) 6.24 and 6.03 (AB, *J* 11Hz, 2H, C-6,7), 5.09 (t, *J* 7Hz, 1H, C-24), 5.05 (m, 1H, C-19), 4.82 (m, 1H, C-19), 3.95 (t of t, *J* 7.4 and 3.7Hz, 1H, C-3), 1.68 (s, 3H, C-26), 1.60 (s, 3H, C-27), 0.94 (d, *J* 6Hz, 3H, C-21), 0.54 (s, 3H, C-18); *m/e* (relative intensity) 382 (M⁺, 24), 349 (8), 253 (6), 136 (100), 118 (78), 79 (41), 55 (30); M⁺, *m/e*, found: 382.3229 calc. for C₂₇H₄₂O: 382.3236; homogeneous on t.l.c. (*R_F* = 0.36, 20% ethyl acetate in Skellysolve B) and g.l.c. (*t_R* = 6.7 and 7.4min for the pyro and isopyro product respectively).

25-Dehydrocholecalciferol. 25-dehydrocholecalciferol 3-acetate (4.9mg) was saponified as described above for the preparation of 24-dehydrocholecalciferol. The resulting oil was purified by preparative t.l.c. (silica gel, with 20% ethyl acetate in Skellysolve B as developing solvent). The major band was eluted with ethyl acetate to give 2.2mg (50%) of 25-dehydrocholecalciferol as an oil: u.v. (ethanol) $\lambda_{\max}$. 265nm; n.m.r. (270MHz) δ (p.p.m.) ([²H]chloroform) 6.24 and 6.04 (AB, *J* 12Hz, 2H, C-6,7), 5.05 (m, 1H, C-19), 4.82 (m, 1H, C-19), 4.69 (s, 1H, C-26), 4.66 (s, 1H, C-26), 3.95 (m, 1H, C-3), 1.72 (s, 3H, C-27), 0.93 (d, *J* 6Hz, 3H, C-21), 0.54 (s, 3H, C-18); *m/e* (relative intensity) 382 (M⁺, 26), 349 (5), 253 (5), 136 (100), 118 (78), 69 (18), 55 (30); homogeneous on t.l.c. (*R_F* = 0.36; 20% ethyl acetate in Skellysolve B) and g.l.c. (*t_R* = 6.5 and 7.2min for the pyro and isopyro derivatives); high-resolution mass spectrum: found: 382.3233; calc. for C₂₇H₄₂O: 382.3236.

Preparation of 25-fluoro[3-³H]cholecalciferol

Radioactively labelled 25(F)D₃ was prepared from 25(OH)[3-³H]D₃ by the procedure used to convert 25(OH)D₃ into 25(F)D₃ (Onisko *et al.*, 1977). This involved selective acetylation (acetic anhydride/pyridine) of 25(OH)[3-³H]D₃ (1.5mg, 30Ci/mol), fluorination of the resulting 3 β -acetate with diethylaminosulphur trifluoride (in dichloromethane for 15min at -78°C), hydrolysis (KOH/methanol) followed by purification by t.l.c. (silica-gel plate 750 μ m thick, 30% ethyl acetate/Skellysolve B). The synthetic product showed a u.v.-absorption maximum at 265nm (in ethanol). A specific radioactivity of 32Ci/mol and an overall yield from 25(OH)D₃ of

Fig. 2. Co-chromatography of 25-fluoro[3-³H]cholecalciferol with authentic 25(F)D₃ on h.p.l.c.

25-Fluoro[3-³H]cholecalciferol was diluted with authentic 25(F)D₃ then analysed by h.p.l.c. as described in the Experimental section. The left ordinate represents the output of the u.v. detector (254 nm) of the instrument (—). The right ordinate represents the radioactivity per fraction (bar graph).

49% were calculated from the absorbance assuming ϵ_{265} 18000 litre·mol⁻¹·cm⁻¹. Purity was established by h.p.l.c. (Fig. 2), with a 0.45 cm × 25 cm column (Zorbax, Dupont) operated at 6.9 MPa (1000 lb/in²) and a flow rate of 1.5 ml/min of propan-2-ol/hexane (1:99, v/v). Solvents were evaporated from the column fractions (0.5 ml) and the residues were redissolved in toluene-based radioactivity-counting solution {2 g of PPO [2,5-diphenyloxazole] and 0.1 g of POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene]/litre of toluene} for liquid-scintillation counting in a Packard Tri-Carb model 3375 instrument.

Bioassays and metabolism experiments

Animals. Male albino weanling rats were obtained from the Holtzman Company (Madison, WI, U.S.A.). They were housed in overhanging wire cages and fed on the vitamin D-deficient, low-calcium (0.02%) normal-phosphorus (0.3%) diet of Suda *et al.* (1970) for 2.5–3 weeks before their use in bioassay and metabolism experiments.

Assay for inhibition of 25-hydroxylation. Vitamin D-deficient rats (see above) were dosed intrajugularly under light ether anaesthesia with either ethanol (0.05 ml, normal controls) or 0.05 ml of ethanol containing graded amounts of Δ^{24} -D₃, Δ^{25} -D₃ or 25(F)D₃. After 2 h, each animal received 1.0 μ Ci

of [3-³H]cholecalciferol (7.9 Ci/mmol) in 0.05 ml of ethanol by the same route. The animals were killed 4 h after the second dose and the 25(OH)[3-³H]D₃ in serum (see Table 1 below) was measured by extraction of the serum by using the method of Bligh & Dyer (1959), chromatography of the extract on Sephadex LH-20 (Holick & DeLuca, 1971) and analysis of the column fractions by liquid-scintillation counting.

Intestinal-calcium-transport and bone-calcium-mobilization assays. Rats maintained as described above were divided randomly into groups of six animals, then dosed intrajugularly under light ether anaesthesia. Doses (see Table 2 below for material and amount) were prepared by dissolving the appropriate substance in ethanol (0.05 ml/rat). The dose protocol (compound, amount, time) is detailed in Tables 2 and 3 below. The animals were killed by decapitation and their blood and duodena were obtained. Bone-calcium mobilization was determined by measuring serum calcium concentrations; 0.1 ml portions of serum were diluted with 1.9 ml of 0.1% aqueous LaCl₃, and calcium was measured by atomic absorption with a Perkin-Elmer model 403 instrument. Intestinal calcium transport was determined by using the everted-gut-sac technique of Martin & DeLuca (1969). For ⁴⁵Ca radioactivity counting, portions from the serosal and mucosal media were spotted on filter-paper discs, dried, and placed in 20 ml counting vials containing 10 ml of toluene counting solution.

Metabolism of Δ^{24} -D₃ and Δ^{25} -D₃. Vitamin D-deficient rats (see above) were divided into groups of three animals, then dosed intrajugularly under ether anaesthesia with either ethanol (0.05 ml) or ethanol (0.05 ml) containing Δ^{24} -D₃ (50 μ g), Δ^{25} -D₃ (50 μ g) or vitamin D₃ (50 ng). At 20 h after dosing, the animals were killed by jugular exsanguination under light ether anaesthesia. The collected blood from rats of the same group was pooled, and the resulting serum was analysed for 1 α ,25(OH)₂D₃ content by the method of Eisman *et al.* (1976). This assay involves extraction of serum, chromatography of the extract on Sephadex LH-20 and h.p.l.c. Determination of 1 α ,25(OH)₂D₃ content in the final chromatographic fraction was by a competitive receptor-binding assay performed on each sample in triplicate.

Metabolism of 25(F)[3-³H]D₃. Vitamin D-deficient rats (see above) were weighed (81 $\pm$ 4 g, mean $\pm$ s.d., n = 4), then half of the animals were bilaterally nephrectomized. Each rat received a dose of 25(F)[3-³H]D₃ (15 μ g, 32 Ci/mol) in 0.05 ml of ethanol via intrajugular injection under ether anaesthesia. (The nephrectomized rats were dosed 5–15 min after surgery.) One normal and one nephrectomized animal were killed by jugular exsanguination at 10 and 20 h after dosing. Blood samples were centrifuged to give serum. Entire small intestines were rinsed with 30 ml of 0.9% (w/v) aqueous NaCl and 30% (w/v) aqueous homogenates of intestine were prepared.

Serum samples were extracted as described by Bligh & Dyer (1959); intestinal homogenates were extracted by the method of Lund & DeLuca (1966). The extracts were chromatographed on a column (2cm×28cm) of Sephadex LH-20 (Holick & DeLuca, 1971). The columns were eluted with chloroform/Skellysolve B (65:35, v/v); 70 fractions (3.2ml each) were collected. The columns were then washed with 150ml of chloroform/Skellysolve B (3:1, v/v) and re-equilibrated with 100ml of the original column solvent. Column recovery of radioactivity was $88 \pm 11\%$ (mean $\pm$ s.d., $n = 8$). None of the column washes contained more than 2% of the applied radioactivity.

Portions (1.0ml) of each column fraction were

Fig. 3. Sephadex LH-20 chromatograms of synthetic 25-fluoro[3- 3 H]cholecalciferol and serum extracts from either an intact or anephric rat given 25-fluoro[3- 3 H]-cholecalciferol

Vitamin D-deficient rats, either intact or nephrectomized, were administered 25-fluoro[3- 3 H]cholecalciferol (15 μ g, 32 Ci/mol). After 10h later the animals were killed, and serum samples were extracted then chromatographed on Sephadex LH-20 as described in the Experimental section. Chromatograms of serum extracts from intact (top panel) and anephric (middle panel) animals are shown. The bottom panel represents the chromatogram of synthetic 25(F)D₃. The ordinate represents the radioactivity per fraction based on the extraction of equivalent volumes of serum. The ordinate is expanded by a factor of 100 for fractions 20–70. Peak F-I represents 25(F)D₃, the peak-F-II component was not identified, and peak F-III contains 1 α (OH)25(F)D₃ (see Fig. 4).

stored under N₂ in the freezer for subsequent h.p.l.c. The remainder of each column fraction was analysed for 3 H after evaporating solvents and redissolving the residue in toluene counting solution. Calculations for metabolite concentration in serum (Table 3) assume that the total serum volume represents 3% of the body weight.

Selected radioactive peaks from the Sephadex LH-20 columns were further analysed by h.p.l.c. on a 0.4cm×30cm column (μ Porasil; Waters), which was operated at 3.45 MPa (500lb/in²). This gave a flow rate of 2.0ml of propan-2-ol/hexane (1:19, v/v)/min. Appropriate Sephadex LH-20 column fractions (see Fig. 3) were pooled, solvents were evaporated, the residues were redissolved in column solvent, and a mixture of standard fluorinated analogues was added before injection. Fractions (50, 0.5ml each) were collected after each sample injection; each was analysed for 3 H as for the Sephadex LH-20 column fractions. H.p.l.c. recovery of radioactivity ranged from 80 to 90%.

Results

The ability of 25(F)D₃, Δ^{24} -D₃ and Δ^{25} -D₃ to inhibit the 25-hydroxylation of cholecalciferol *in vivo* is demonstrated by the data of Table 1. 25(OH)D₃ is the major circulatory form of cholecalciferol and is present in the blood of normal control rats in high concentration. (Approx. 10% of a dose of [3 H]-cholecalciferol is found in blood as 25(OH)[3 H]D₃, 4h after administration.) When, however, the animals are predosed with 25(F)D₃, Δ^{24} -D₃ or Δ^{25} -D₃, the serum concentration of 25(OH)D₃ is markedly decreased. For example, a 50 μ g dose of any of the three

Table 1. Inhibition *in vivo* of 25-hydroxylation of cholecalciferol by 25(F)D₃, Δ^{24} -D₃ or Δ^{25} -D₃

The analogues were administered to vitamin D-deficient rats in ethanol (0.05ml) by intrajugular injection. After 2h, each animal received a dose of [3 - 3 H]cholecalciferol by the same route. At 4h after this dose, the serum content of 25(OH)[3 - 3 H]D₃ was measured as described in the Experimental section.

Analogue dose (μ g)	Serum 25-OH-D ₃ concentration* in rats given:		
	25(F)D ₃	Δ^{24} -D ₃	Δ^{25} -D ₃
0	10.4 $\pm$ 0.6†	—	—
0.5	5.8	1.9	—
5.0	1.9	0.80	0.90
50.0	0.44	0.59	0.66

* Expressed as a percentage of total dose of administered [3 - 3 H]cholecalciferol present in serum as 25(OH)D₃.

† Mean $\pm$ s.e.m., $n = 3$; each of the other values in the Table represent the findings for a single animal.

compounds decreased the conversion of [^3H]cholecalciferol into $25(\text{OH})[^3\text{H}]\text{D}_3$ by more than a factor of 10. Furthermore, increasing doses of either $25(\text{F})\text{D}_3$, $\Delta^{24}\text{-D}_3$, or $\Delta^{25}\text{-D}_3$ progressively decreased serum $25(\text{OH})\text{D}_3$ content (Table 1).

On the basis of this inhibition of the 25-hydroxylation of vitamin D, one would expect the compounds to block, or at least diminish, the biological responses of cholecalciferol. Relevant data on the effect of $25(\text{F})\text{D}_3$, $\Delta^{24}\text{-D}_3$, and $\Delta^{25}\text{-D}_3$ on the intestinal-calcium-transport and bone-calcium-mobilization responses of vitamin D-deficient rats given cholecalciferol are summarized in Table 2. None of the

compounds (groups 3, 4 and 5, Table 2) antagonized the normal physiological responses elicited by cholecalciferol. Intestinal calcium-transport ratios and serum calcium values for these groups were significantly elevated compared with (–) control rats (dosed with ethanol only, Group 1), and were even greater than those for (+) control rats (dosed with cholecalciferol only, Group 2).

The possibility, suggested by these results, that the analogues themselves might be active, is confirmed by the data of Table 2 (Groups 6, 7 and 8) and those of Table 3. The administration of high doses ($50\mu\text{g}/\text{animals}$) of $25(\text{F})\text{D}_3$ (Group 6), $\Delta^{24}\text{-D}_3$ (Group 7)

Table 2. *Biological assay of $25(\text{F})\text{D}_3$, $\Delta^{24}\text{-D}_3$, and $\Delta^{25}\text{-D}_3$*

Dose 1 was administered to vitamin D-deficient rats 2h before Dose 2. The rats were killed 20h after Dose 2 and the biological responses were measured as detailed in the Experimental section. Additional abbreviation used: D_3 , cholecalciferol (vitamin D_3).

Group	Dose 1	Amount	Dose 2	Amount	Intestinal Ca^{2+} transport* (mean $\pm$ S.E.M.†)	Bone Ca^{2+} mobilization† (mean $\pm$ S.E.M.†)
1	Ethanol	0.05 ml	Ethanol	0.05 ml	2.1 ± 0.2 (6)	4.5 ± 0.1 (6)
2	Ethanol	0.05 ml	D_3	$0.05\mu\text{g}$	4.9 ± 0.2 (6)	5.0 ± 0.1 (6)§
3	$25(\text{F})\text{D}_3$	$50\mu\text{g}$	D_3	$0.05\mu\text{g}$	5.8 ± 0.4 (6)	6.1 ± 0.3 (6)
4	$\Delta^{24}\text{-D}_3$	$50\mu\text{g}$	D_3	$0.05\mu\text{g}$	6.0 ± 0.6 (6)	6.1 ± 0.1 (6)
5	$\Delta^{25}\text{-D}_3$	$50\mu\text{g}$	D_3	$0.05\mu\text{g}$	6.1 ± 0.4 (6)	5.9 ± 0.2 (6)
6	Ethanol	0.05 ml	$25(\text{F})\text{D}_3$	$50\mu\text{g}$	5.0 ± 0.4 (6)	6.1 ± 0.3 (6)
7	Ethanol	0.05 ml	$\Delta^{24}\text{-D}_3$	$50\mu\text{g}$	4.7 ± 0.4 (6)	5.8 ± 0.3 (6)
8	Ethanol	0.05 ml	$\Delta^{25}\text{-D}_3$	$50\mu\text{g}$	5.4 ± 0.2 (6)	5.9 ± 0.3 (6)

* Serosal $^{45}\text{Ca}/\text{mucosal } ^{45}\text{Ca}$.

† Numbers in parentheses represent numbers of rats.

‡ Serum calcium (mg/100ml).

§ Significant difference from Group 1 by Student's t test, $P < 0.01$; all other entries for groups 2–8 differ from control group 1, $P < 0.001$.

Table 3. *Biological activity of $25(\text{F})\text{D}_3$, $\Delta^{24}\text{-D}_3$, and $\Delta^{25}\text{-D}_3$*

Vitamin D-deficient rats were dosed with the indicated compound by intrajugular injection. After 20h, the animals were killed and the intestinal and bone responses were measured as detailed in the Experimental section. Additional abbreviation used: D_3 , cholecalciferol (vitamin D_3).

Group	Dose	Amount	Intestinal Ca^{2+} transport* (mean $\pm$ S.E.M.†)	Bone Ca^{2+} mobilization† (mean $\pm$ S.E.M.†)
1	Ethanol	0.05 ml	2.1 ± 0.2 (6)	4.6 ± 0.2 (6)
2	D_3	$0.05\mu\text{g}$	4.9 ± 0.4 (6)§	5.8 ± 0.2 (6)§
3	$25(\text{F})\text{D}_3$	$5\mu\text{g}$	3.8 ± 0.3 (6)§	5.6 ± 0.1 (6)§
4	$\Delta^{24}\text{-D}_3$	$5\mu\text{g}$	3.4 ± 0.3 (6)§	5.2 ± 0.3 (6)
5	$\Delta^{25}\text{-D}_3$	$5\mu\text{g}$	3.8 ± 0.3 (6)§	4.8 ± 0.2 (6)
6	$25(\text{F})\text{D}_3$	$0.5\mu\text{g}$	3.2 ± 0.3 (6)	4.7 ± 0.1 (6)
7	$\Delta^{24}\text{-D}_3$	$0.5\mu\text{g}$	2.6 ± 0.2 (6)	4.4 ± 0.1 (6)
8	$\Delta^{25}\text{-D}_3$	$0.5\mu\text{g}$	2.2 ± 0.3 (5)	4.6 ± 0.1 (6)

* Serosal $^{45}\text{Ca}/\text{mucosal } ^{45}\text{Ca}$.

† Serum calcium (mg/100ml).

‡ Numbers in parentheses represent numbers of rats.

§ Significant difference from Group 1 by Student's t test ($P < 0.01$).

|| Significant difference from Group 1 ($P < 0.005$); all other entries were not significantly different from Group 1 ($P < 0.05$).

and Δ^{25} -D₃ (Group 8) to vitamin D-deficient rats produced significant stimulation of intestinal calcium transport and bone mineral resorption (serum Ca) in all cases (Table 2). At lower doses (5 μ g, Table 3) only 25(F)D₃ significantly elevated serum calcium, whereas intestinal calcium transport was stimulated by all three compounds. At the lowest dose (0.5 μ g, Table 3) no significant bone response was observed, and only 25(F)D₃ exhibited a moderate activity in intestine. The magnitude of the responses caused by 25(F)D₃, Δ^{24} -D₃ and Δ^{25} -D₃, when compared with that of a physiological dose of cholecalciferol (0.05 μ g) establishes the potency *in vivo* of 25(F)D₃ to be 100–300 times lower than that of cholecalciferol, whereas Δ^{24} -D₃ and Δ^{25} -D₃ are estimated to be $\sim$ 300–1000 times less active than cholecalciferol.

The biological activity of the two dehydro compounds is accounted for by their conversion, *in vivo*, into $1\alpha,25(\text{OH})_2\text{D}_3$. At 20 h after administration of a 50 μ g dose of Δ^{24} -D₃ or Δ^{25} -D₃ to vitamin D-deficient rats, the concentration of $1\alpha,25(\text{OH})_2\text{D}_3$ in serum was 110 pg/ml and 180 pg/ml respectively (Table 4). Under these conditions, untreated (ethanol-dosed) control animals had undetectable (<3 pg/ml of serum) amounts of circulating $1\alpha,25(\text{OH})_2\text{D}_3$, whereas in rats given cholecalciferol (0.05 μ g) serum concentrations of $1\alpha,25(\text{OH})_2\text{D}_3$ reached values of 300 pg/ml (Table 4). Although the substance produced *in vivo* from both Δ^{24} -D₃ and Δ^{25} -D₃ was not isolated and specifically characterized, it is safe to conclude that it is indeed $1\alpha,25(\text{OH})_2\text{D}_3$, since the assay used (Eisman *et al.*, 1976) requires (1) co-migration with $1\alpha,25(\text{OH})_2\text{D}_3$ on Sephadex LH-20 and h.p.l.c., and (2) the displacement of bound $1\alpha,25(\text{OH})_2[^3\text{H}]\text{D}_3$ from its highly specific intestinal receptor (Kream *et al.*, 1977).

Table 4. Concentrations of $1\alpha,25(\text{OH})_2\text{D}_3$ in serum of vitamin D-deficient rats after administration of Δ^{24} -D₃, Δ^{25} -D₃, cholecalciferol or ethanol

The compounds were administered to vitamin D-deficient animals in ethanol (0.05 ml) by intrajugular injection. After 20 h, serum $1\alpha,25(\text{OH})_2\text{D}_3$ concentrations were measured as described in the Experimental section.

Compound	Dose (μ g)	Serum $1\alpha,25(\text{OH})_2\text{D}_3$ (pg/ml)
Ethanol	—	<3†
24-Dehydrocholecalciferol	50	110 $\pm$ 40
25-Dehydrocholecalciferol	50	180 $\pm$ 20
Cholecalciferol	0.05	300 $\pm$ 100

* Values are expressed as the mean $\pm$ S.E.M. for triplicate determinations.

† Undetectable in assay.

The relatively higher potency *in vivo* of 25(F)D₃ (as compared with Δ^{24} -D₃ and Δ^{25} -D₃; see Tables 2 and 3) suggested that this analogue might also be subject to metabolic activation. To investigate this question directly we prepared ³H-labelled 25(F)D₃ by fluorination of 25-hydroxy[3-³H]cholecalciferol 3-acetate. Synthetic 25-fluoro[3-³H]cholecalciferol was characterized by its u.v.-absorption spectrum (λ_{max} , 265 nm) and co-migration with authentic 25(F)D₃ on analytical h.p.l.c. (see Fig. 2). These data and its known method of preparation (Onisko *et al.*, 1977) assure the authenticity and purity of the radioactively labelled substance.

When the 25-fluoro[3-³H]cholecalciferol (15 μ g/animal) was administered to vitamin D-deficient rats, and serum and intestinal extracts were chromatographed on Sephadex LH-20, two minor radioactive peaks more polar than 25(F)D₃ (peak F-I) were observed, namely peaks F-II and F-III, as shown in

Fig. 4. Chromatography of peak-F-III components from intact or anephric rats on h.p.l.c., and comparison with synthetic standards

Portions of the column fractions containing peak-F-III components obtained by chromatography of serum extracts on Sephadex LH-20 (see Fig. 3) were pooled (fractions 37–51 for the sample from the intact rat, fractions 33–47 for the sample from the anephric rat). The pooled fractions were analysed by h.p.l.c. as described in the Experimental section. The synthetic standards were 25(F)D₃ (2), 24R(OH)25(F)D₃ (3) and $1\alpha(\text{OH})25(\text{F})\text{D}_3$ (4). The solvent front in the bottom panel is indicated by 1.

Table 5. *Distribution of peak-F-I and -F-III components in blood and intestine of intact and anephric rats*
 25-Fluoro[3-³H]cholecalciferol (15 μ g, 32Ci/mol) was administered to either intact or nephrectomized vitamin D-deficient rats. The animals were killed at the times indicated and serum samples and intestinal homogenates were extracted and chromatographed on Sephadex LH-20 as described in the Experimental section.

Time (h after dose)	Tissue examined	Distribution (percentage of the total dose administered)			
		Peak-F-I* component		Peak-F-III components	
	Rat...	Intact	Anephric	Intact†	Anephric‡
10	Serum	8.0	14.0	0.26	0.19
20	Serum	6.7	9.8	0.33	0.20
10	Intestine	1.3	2.6	0.06	0.04
20	Intestine	1.1	2.1	0.08	0.09

* Identified as 25(F)D₃.

† Found by h.p.l.c. to contain 1 α (OH)25(F)D₃ in addition to the unknown metabolite F-III_a.

‡ Identified as metabolite F-III_a by h.p.l.c.

Fig. 3 for the serum extract. Both peaks F-II and F-III were also present in extracts of serum and intestine of a bilaterally nephrectomized animal.

The peak-F-III region isolated by Sephadex LH-20 chromatography of the serum extract of either an intact or anephric rat was then examined by analytical h.p.l.c. (Fig. 4). Chromatography of peak F-III from an intact rat resulted in resolution of two components, F-III_a and F-III_b, present in roughly equal amounts. Component F-III_b co-migrated exactly with 1 α (OH)25(F)D₃, but peak F-III_a did not co-migrate with any of the available synthetic fluoro vitamin D analogues; its identity remains unknown. The synthesis of 1 α (OH)25(F)D₃ (component F-III_b) in the kidney was established by h.p.l.c. analysis of peak F-III from an anephric rat showing only the presence of component F-III_a.

Quantitative distribution data for peaks F-I and F-III derived from the Sephadex LH-20 column profiles are summarized in Table 5. (Peak F-II could not be quantified accurately because it represents a small shoulder after the large F-I peak.) In intact animals, the concentrations of 25(F)D₃ (peak F-I) in both serum and intestine decreased with time; amounts of peak-F-III material remained essentially constant. The same is true for anephric animals, but the significantly higher amounts of 25(F)D₃ in intestine and serum and at both time points are noteworthy. Peak-F-III material never amounted to more than 0.33% of the dose, and the quantitative differences between normal and anephric animals are slight. It should be recalled, however, that component F-III represents a mixture (about 1:1) of two components [1 α (OH)25(F)D₃ and an unknown component, F-III_a] in normal animals, but a single species (component F-III_a) in the case of anephric rats. In terms of absolute quantities, this means that the concentration of 1 α (OH)25(F)D₃ in the blood of an intact animal reaches a value of 7.1 ng/ml of serum 10h after a 15 μ g injection of 25(F)D₃.

Discussion

Although all these C-25-blocked analogues [25(F)D₃, Δ^{24} -D₃ and Δ^{25} -D₃] effectively inhibited the conversion *in vivo* of cholecalciferol into 25(OH)D₃ (Table 1), none was capable of antagonizing the biological responses induced by cholecalciferol in vitamin D-deficient animals (Table 2). The demonstration that the analogues possess inherent biological activity (Tables 2 and 3) explains and reconciles these seemingly disparate results. Considering the structures of the three compounds it appeared reasonable to assume that their activity was not the result of direct cellular action, but that, like cholecalciferol itself, they were subject to metabolism to more active forms. For the case of the two dehydro analogues, Δ^{24} -D₃ and Δ^{25} -D₃, we have shown that the active form is 1 α ,25(OH)₂D₃ (Table 4).

The required conversion *in vivo* of the dehydro analogues into 1 α ,25(OH)₂D₃ is obviously a low-yield and inefficient process, as evidenced by the low potency of either Δ^{24} -D₃ or Δ^{25} -D₃ compared with cholecalciferol (Table 3), but the amounts of 1 α ,25(OH)₂D₃ accumulating in blood in response to analogue administration (Table 4) are quite adequate to account for their biological activity. We presume that metabolism of Δ^{24} -D₃ and Δ^{25} -D₃ involves an initial reduction to cholecalciferol, followed by the normal two-step hydroxylation sequence. Direct reduction of the side-chain double bond of Δ^{24} -D₃ and Δ^{25} -D₃ is certainly conceivable and, based on analogous conversions, probably to be expected. Desmosterol is reduced to cholesterol by the rat liver sterol Δ^{24} -reductase (Avigan & Steinberg, 1961). The enzyme has broad substrate specificity, since lanosterol is reduced at a rate comparable with that observed for desmosterol (Avigan *et al.*, 1963). Cell-free liver preparations are also capable of converting 25-dehydrocholesterol to cholesterol, but at a rate much slower than that for desmosterol (Steinberg &

Avigan, 1969). An alternate possibility, namely that reduction does not occur, and that $1\alpha,25(\text{OH})_2\text{D}_3$ is synthesized *in vivo* from cholecalciferol present as a trace contaminant in our preparations of $\Delta^{24}\text{-D}_3$ or $\Delta^{25}\text{-D}_3$, is highly unlikely; the dehydro compounds are prepared from $25(\text{OH})\text{D}_3$, and analysis (g.l.c., mass spectrometry) of both the starting material and the products give no evidence of cholecalciferol contamination.

Metabolic activation of $25(\text{F})\text{D}_3$ also involves 1α -hydroxylation. Our results (Table 5, Figs. 3 and 4) demonstrate the conversion *in vivo* of $25(\text{F})\text{D}_3$ into $1\alpha(\text{OH})25(\text{F})\text{D}_3$. The metabolite was characterized by chromatographic comparisons with synthetic material, the high potency *in vivo* of which (with respect to stimulation of intestinal calcium transport and bone calcium mobilization in the rat) has been established (Napoli *et al.*, 1978). Although the yield of $1\alpha(\text{OH})25(\text{F})\text{D}_3$ is low, the amounts of metabolite produced over the time course of the assay (e.g. 7.1 ng/ml of serum after 10h, on the basis of the data of Table 5 and Fig. 4) appear adequate, in view of the results of Napoli *et al.* (1978), to account for the biological response observed. It is unlikely, in any case, that the other metabolite fractions, i.e. those under peaks F-II and F-III_a (Figs. 3 and 4), are significant contributors to activity; their synthesis in the absence of kidney tissue implies that they are not 1-hydroxylated (an extrarenal 1-hydroxylase for vitamin D compounds has, as yet, not been encountered). Given the importance of a 1α -hydroxy function for expression of activity, these unknown metabolites of $25(\text{F})\text{D}_3$ are not expected to induce a significant biological response at the concentrations measured (Table 5, Figs. 3 and 4). C-1 hydroxylation of $25(\text{F})\text{D}_3$ therefore accounts, at least in large measure, for the biological activity observed for $25(\text{F})\text{D}_3$ and explains its failure to antagonize the action of cholecalciferol, even though $25(\text{F})\text{D}_3$ is by far a more potent inhibitor of the 25-hydroxylation of vitamin D than the known vitamin D antagonist, 25-azacholecalciferol (Onisko *et al.*, 1979a).

It is conceivable that the action of $1\alpha(\text{OH})25(\text{F})\text{D}_3$ requires further metabolism [e.g. to $1\alpha,24\text{R}(\text{OH})_2\text{-}25(\text{F})\text{D}_3$], but no species more polar than the peak-F-III component was detected in our chromatograms. The absence of $1\alpha(\text{OH})25(\text{F})\text{D}_3$ in nephrectomized animals strongly suggests that 1α -hydroxylation of $25(\text{F})\text{D}_3$ is catalysed by the kidney 1-hydroxylase, for which $25(\text{OH})\text{D}_3$ and also $24,25(\text{OH})_2\text{D}_3$ are the normal substrates *in vivo*. If so, our original assumption that $25(\text{F})\text{D}_3$, by virtue of the missing C-25 hydroxy substituent, would not be a substrate for the 1-hydroxylase is evidently not valid. That assumption was based on previous studies

of the chick kidney enzyme, which had clearly shown that vitamin D compounds lacking the C-25 hydroxy function, e.g. cholecalciferol itself, or even $24\text{R}(\text{OH})\text{D}_3$, are not 1α -hydroxylated under conditions that rapidly convert their 25-hydroxy analogues (Gray *et al.*, 1972; Tanaka *et al.*, 1977). An electro-negative fluorine substituent at C-25 may, of course, render $25(\text{F})\text{D}_3$ a somewhat better substrate than cholecalciferol itself, and this factor, combined with high substrate concentrations (e.g. 50 $\mu\text{g}/\text{animal}$), and possibly a somewhat broader substrate specificity of the rat enzyme, can account for the observed low-yield 1α -hydroxylation of $25(\text{F})\text{D}_3$. These questions can be addressed directly by comparisons *in vitro* of the chick and rat 1α -hydroxylase systems.

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