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1α-Hydroxyvitamin D_2 is Less Toxic than 1α-Hydroxyvitamin D_3 in the Rat (42028)

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Abstract. An LD_{50} of 0.2 mg/kg body wt has been determined for 1α-hydroxyvitamin D_3 in the rat. In comparison, the LD_{50} for 1α-hydroxyvitamin D_2 is between 3.5 and 6.5 mg/kg. In terms of chronic toxicity, 1α-hydroxyvitamin D_3 at a dose of 5 μg/kg/day causes death of one-half the animals in a 4-week period. On the other hand, 20 μg/kg/day of 1α-hydroxyvitamin D_2 is required to induce similar toxicity. The body weight record and renal calcium accumulation during chronic treatment support the above conclusion. It therefore appears that 1α-hydroxyvitamin D_2 is between 5 and 15 times less toxic than 1α-hydroxyvitamin D_3. This surprising result prompted a reexamination of the relative biological activity of 1α-hydroxyvitamin D_2 and 1α-hydroxyvitamin D_3. Both compounds are equally potent in the stimulation of intestinal calcium transport, bone calcium mobilization, in the elevation of serum phosphorus, and in the healing of rickets in the rat. The reason for lower toxicity of 1α-hydroxyvitamin D_2 is unknown. The results suggest that 1α-hydroxyvitamin D_2 might represent a therapeutically superior compound. © 1985 Society for Experimental Biology and Medicine.

The discovery that vitamin D must be metabolically activated by 25-hydroxylation followed by 1-hydroxylation to yield the final vitamin D hormone, 1α,25-dihydroxyvitamin D_3 ($1,25\text{-}(OH)_2D_3$) has resulted in a major interest in the chemical synthesis of analogs of $1,25\text{-}(OH)_2D_3$ (1, 2). Of the many analogs synthesized, 1α-OH-D_3 has proved to be the most important from a practical point of view and from a therapeutic point of view (3). This compound appears to be approximately one-half as active as $1,25\text{-}(OH)_2D_3$ in stimulating intestinal calcium transport, in the mobilization of calcium from bone, and in the mineralization of the skeleton (4). 1α-OH-D_3 must be 25-hydroxylated *in vivo* before it can exert its biological activity on the target organs (5, 6).

Chemical synthesis of 1α-OH-D_2 had previously been achieved and the compound was shown to have activity similar to 25-OH-D_3 but appeared to be somewhat less active in the mobilization of calcium from bone (7, 8). The limited availability of this compound because of the inadequate synthetic method has limited investigation of its potential use. More recently a chemical synthesis for 1α-hydroxylated vitamin D compounds has been devised that is particularly applicable to the synthesis of 1α-OH-D_2 (9, 10). This synthesis has made available sizable quantities of this compound for biological testing. One of the surprising results is that this compound is much less toxic than 1α-OH-D_3 and yet appears to be almost equally active in the classical responses of vitamin D-deficient rats. This paper reports those results.

Materials and Methods. *Animals.* For toxicity studies, male rats (approximately 200 g) were purchased from the Holtzman Company (Madison, Wisc.). They were placed on a Purina Laboratory Chow diet (Ralston Purina, St. Louis, Mo.).

For experiments dealing with a biological potency of the compounds, weanling male rats from Holtzman rats were fed either a vitamin D-deficient diet containing 0.02% Ca and 0.3% P (11) or a high calcium (1.2%), low phosphorus (0.1%) diet that produces rickets (12). The low calcium diet was used to study intestinal calcium transport and bone calcium mobilization responses whereas the high calcium, low phosphorus diet was used to examine the antirachitic activity of the compounds.

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TABLE I. DETERMINATION OF LD_{50} FOR 1α-OH-D_2

	Dose (mg/kg)	Survival ratio
1α-OH-D_2	10	0/6[b]
	6.5	3/6[a]
	3.5	3/6[a]
1α-OH-D_3	1.5	0/6[b]
	1.0	0/6[b]
	0.5	1/6[a]
	0.2	3/6[a]
	0.1	5/6[a]

[a] Significantly different from *b*; $P < 0.01$–0.001. Survival was assessed at 240 hr after the dose.

Chemicals. 1α-OH-D_3 and 1α-OH-D_2 were synthesized according to the method of Paaren *et al.* (9, 10). Pure crystalline 1α-OH-D_2 and 1α-OH-D_3 had the expected physical constants previously reported (8–10, 13). 1,25-$(OH)_2D_3$ was generously provided by the Hoffmann–LaRoche Company (Nutley, N.J.). The concentration of each compound was determined by ultraviolet absorption spectra taken in ethanol with a Beckman Model DK recording spectrophotometer. Aliquots of standard solutions made in this fashion were dissolved in cottonseed–soybean oil (Wesson) and used for oral dosage (for toxicity tests). For determination of biological activity, the compounds were dissolved in ethanol:propylene glycol (5:95) and given intraperitoneally.

Experimental Design. For determination of LD_{50}, oral doses of the 1α-OH-D compounds were given by stomach tube in Wesson oil as a single dose. The deaths occurring as a result of dosage were noted. The results are calculated on the basis of the dosage per kilogram body weight required to cause death of 50% of the animals. For chronic toxicity, the indicated doses of the vitamin D compounds dissolved in Wesson oil were prepared. The animals received daily oral doses of the indicated compounds for the period of time designated. The animals were weighed each week and at the conclusion of the experiment they were killed, plasma was taken for determination of calcium concentration, and kidneys were removed for determination of calcium content. In this case, kidneys were ashed at 600°C for a 24-hr period. Calcium content of the ash was determined with an atomic absorption spectrometer (Perkin Elmer Model 403) by dissolving the ash in 0.1 *N* HCl containing 0.1% $LaCl_3$. Serum calcium concentration was determined directly by the same method after dilution in 0.1% $LaCl_3$.

For determination of biological activity, weanling male rats were placed on the low calcium, normal phosphorus diet described above for a period of 3 weeks. At this time, they were given daily intraperitoneal injections of the indicated compounds dissolved in 0.1 ml of 95% propylene glycol, 5% ethanol. Twelve hours after the last dose, the animals were killed and serum calcium and intestinal calcium transport measurements were made as described previously (14, 15). For antirachitic activity determination, the animals were maintained on a low phosphorus, high calcium diet for a period of 3 weeks. At this time they were given daily intraperitoneal injections of the indicated compounds for 7 days. At the end of the 7-day period, the animals were killed and inorganic phosphorus was determined by the method of Chen (16). The rise in serum phosphorus is directly related to the healing of rickets (12). Results were analyzed statistically by unpaired Student's *t* test.

Results. Table I provides the data used to deduce the LD_{50} for 1α-OH-D_3 and 1α-OH-D_2. The LD_{50} for 1α-OH-D_2 appears to be approximately 15 times greater than that for 1α-OH-D_3. Thus by this method, 1α-OH-D_2 is considerably less toxic than 1α-OH-D_3. Chronic toxicity of 1α-OH-D_2 was determined as shown in Table II. 1α-OH-D_2 resulted in intoxication at dosages of 60 μg/kg body wt. However, at doses of 30 μg/kg body wt or below, no intoxication was observed

TABLE II. CHRONIC TOXICITY OF 1α-OH-D_2

Dose (μg/kg/day)	Survival	Hours after dose
300	0/6	48
120	0/6	72
60	4/6	240
30	6/6	240
20	6/6	240
8	6/6	240

for at least 240 hr. Thus, the range of intoxication appeared to be below 30 μg/kg/day. The results in Table III illustrate that for a 4-week period, no intoxication was found with 1α-OH-D_2 at a dose of 5 μg/kg/day. Toxicity did begin to appear at 20 μg/kg/day. In contrast, chronic dosage with 1α-OH-D_3 caused intoxication at 5 μg/kg/day. In this test, therefore, 1α-OH-D_2 appeared to be four to five times less toxic than 1α-OH-D_3. The recording of body weights, serum calcium, and kidney calcium per gram tissue weight supports the reduced intoxication of 1α-OH-D_2 (Figs. 1 and 2) (Table IV). It appears by these measurements that 1α-OH-D_3 is between four and five times more toxic than 1α-OH-D_2.

From these results, it can be argued that 1α-OH-D_2 might be less biologically active than 1α-OH-D_3. A previous comparison with 25-OH-D_2 suggested that 1α-OH-D_2 would be equal in biopotency to 1α-OH-D_3 with the possible exception of bone calcium mobilization (8). The results shown in Table V illustrate that both compounds are equally active and approximately equally active to 1,25-$(OH)_2D_3$ in the stimulation of intestinal calcium transport and in the mobilization of calcium from bone of vitamin D-deficient rats. The rise in serum phosphorus in rats on a low phosphorus diet also suggests that 1α-OH-D_2 and 1α-OH-D_3 are nearly equally potent as antirachitic substances in the rat (Fig. 3). However, at the highest dose, 1α-OH-D_3 appeared more active in elevating serum phosphorus levels. Not shown is that

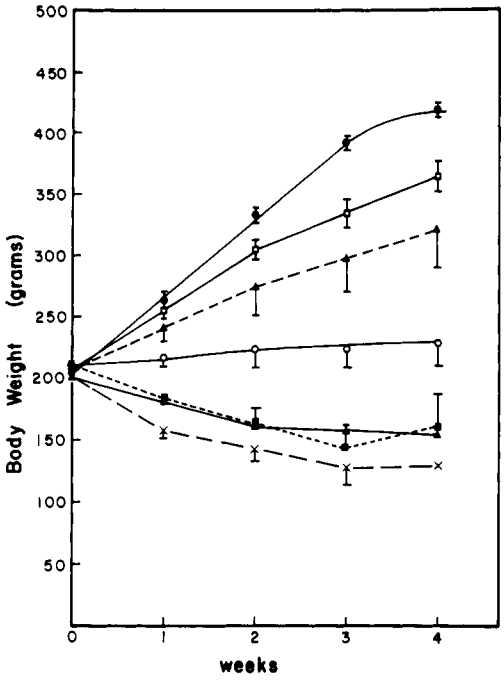

FIG. 1. Weight record of rats given various doses of 1α-OH-D_2 and 1α-OH-D_3. Rats were fed the Ralston Purina Chow diet for the indicated period of 4 weeks. The dosages were given by mouth in Wesson oil. All doses were dissolved in 0.1 ml cottonseed-soybean oil and given either by mouth or gastric tube. The weights were recorded weekly. (●) control (Wesson oil); (□ — □) 1α-OH-D_2, 2.5 μg/kg/day; (▲ --- ▲) 1α-OH-D_2, 5.0 μg/kg/day; (○) 1α-OH-D_3, 2.5 μg/kg/day; (■ --- ■) 1α-OH-D_2, 20 μg/kg/day; (▲ — ▲) 1α-OH-D_3, 5 μg/kg/day; (×) 1α-OH-D_3, 20 μg/kg/day. Vertical bars represent SEM for six rats per group. (□ — □) significantly different from (○) $P < 0.001$. (▲ --- ▲) significantly different from (▲ — ▲) $P < 0.001$.

TABLE III. COMPARISON OF 1α-OH-D_2 AND 1α-OH-D_3 TOXICITY IN RAT

Treatment	Dose μg/kg/day	Deaths 1	2	3	4 Week	Total deaths
1α-OH-D_2	2.5	0	0	0	0	0
	5.0	0	0	0	0	0
	20.0	0	0	0	2	2[b]
1α-OH-D_3	2.5	0	0	0	0	0
	5.0	0	0	1	1	2[b]
	20.0	2	0	1	2	5[a]

Note. There were six rats per group.
[a] Significantly different from all other groups; $P < 0.001$.
[b] Significantly different from unmarked groups; $P < 0.01$.

healing of rachitic epiphyseal plates of these animals was equally induced by these compounds. In studies not reported here, oral doses gave identical results.

Discussion. The present study provides evidence that 1α-OH-D_2 is considerably less toxic than 1α-OH-D_3 in the rat. The most dramatic results are obtained in the determination of an LD_{50} in which 1α-OH-D_2 was found to be approximately 15 times less toxic than 1α-OH-D_3. With chronic doses, 1α-OH-D_2 appeared to be one-fifth as toxic as 1α-OH-D_3. These results are supported by the measurement of serum calcium, body weights, and renal accumulation of calcium.

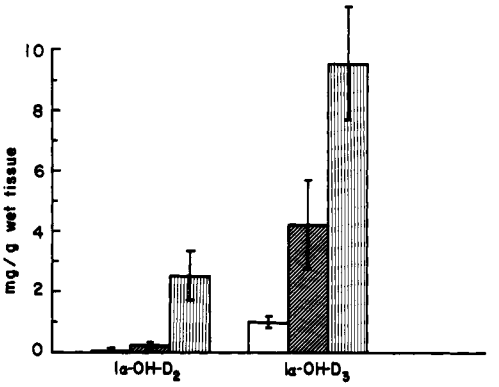

FIG. 2. Kidney calcium content of animals given various levels of 1α-OH-D_2 and 1α-OH-D_3. The calcium content of kidneys resulting from the 4-week chronic intoxication study are recorded. Doses = □ 2.5 μg/kg/day; ▨ 5 μg/kg/day; ▩ 20 μg/kg/day. Vertical bars represent SEM for six rats. 1α-OH-D_2 group differs from 1α-OH-D_3 group at each dosage level, $P < 0.001$.

1α-OH-D_2 appears to be equally antirachitic to 1α-OH-D_3 as revealed by a rise of serum phosphorus in rats on a low phosphorus rachitogenic diet. At the highest dose, 1α-OH-D_3 appeared more active than 1α-OH-D_2 in increasing serum phosphorus. Thus, increased toxicity of 1α-OH-D_3 may be related to this finding since increased serum phosphorus may contribute to nephrocalcinosis. 1α-OH-D_2 is equally active as 1α-OH-D_3 in stimulating intestinal calcium transport and bone calcium mobilization as revealed by a rise in serum calcium in rats on a low calcium diet. The reasons for increased toxicity by 1α-OH-D_3 therefore remains unknown in view of the fact that 1α-OH-D_2 and 1α-OH-D_3 appear to be equally potent in most of the vitamin D responsive systems. The equal biopotency of the two compounds was also found when they were given orally (unpublished results). Thus the difference in toxicity is not likely the result of differential absorption, although this was not tested directly with the high doses. It is entirely possible that at very high doses, 1α-OH-D_3 is metabolized more slowly than 1α-OH-D_2. Thus it would tend to accumulate more readily and result in intoxication. In chicks, D_2 compounds are more rapidly metabolized than are D_3 compounds (17–19), providing support for this idea. However, unlike the rat, the chick responds poorly to physiologic doses of vitamin D_2 as compared to vitamin D_3. For whatever reason, the fact that 1α-OH-D_2 appears to be equally active

TABLE IV. SERUM CALCIUM OF RATS GIVEN GRADED LEVELS OF 1-HYDROXYLATED VITAMIN D COMPOUNDS

Dose (μg/kg/day)	3 Days	2 Weeks	4 Weeks
None	10.6 ± 0.1	10.9 ± 0.2	11.1 ± 0.1
2.5 1α-OH-D_2	13.1 ± 0.4	12.6 ± 0.2	12.9 ± 0.2
5.0 1α-OH-D_2	14.0 ± 0.6	13.1 ± 0.3	13.8 ± 0.5
20.0 1α-OH-D_2	15.5 ± 0.3	13.9 ± 0.2	13.1 ± 0.3[b]
2.5 1α-OH-D_3	14.6 ± 0.3	13.8 ± 0.2	14.0 ± 0.2
5.0 1α-OH-D_3	15.0 ± 0.3	14.2 ± 0.2	13.4 ± 0.3[b]
20.0 1α-OH-D_3	14.8 ± 1.2[b]	14.9 ± 0.5[b]	14.3[a]

Note. There were 6 animals per group and values are reported as means ± SEM except in groups where deaths reduced the numbers to (1)[a], (4)[b]. All values of dosed groups were significantly higher than control, $P < 0.001$. At 2.5 μg dose, serum calcium was higher in the 1α-OH-D_3 group than in the 1α-OH-D_2 group at all times ($P < 0.001$). At the 5.0 μg dose level, the 1α-OH-D_3 group was higher than the 1α-OH-D_2 group at 3 days ($P < 0.05$), but not at 2 and 4 weeks. At 20.0 μg doses, no differences were noted except at 2 weeks where the values in the 1α-OH-D_3 group were higher than in the 1α-OH-D_2 group ($P < 0.01$).

TABLE V. BONE CALCIUM MOBILIZATION (SERUM CALCIUM) AND INTESTINAL CALCIUM TRANSPORT ACTIVITY OF 1α-OH-D_2

Compound	Dose (ng)	Serum calcium (mg/100 ml)	Intestinal calcium S/M
None	—	4.2 ± 0.1	3.3 ± 0.1
1,25-$(OH)_2D_3$	2.5	4.5 ± 0.1	4.9 ± 0.2
	12.5	4.9 ± 0.1	5.9 ± 0.2
	25.0	5.4 ± 0.1	7.5 ± 0.1
1α-OH-D_2	2.5	4.5 ± 0.2	4.8 ± 0.4
	12.5	4.7 ± 0.2	5.0 ± 0.4
	25.0	5.3 ± 0.2	6.0 ± 0.2
1α-OH-D_3	2.5	4.7 ± 0.1	4.1 ± 0.2
	12.5	5.2 ± 0.1	4.4 ± 0.5
	25.0	5.6 ± 0.2	5.9 ± 0.3

Note. Weanling male rats were fed the low (0.02% Ca, 0.3% P) vitamin D-deficient diet for 3 weeks and then given the indicated compounds. There were 6 rats per group and values are means ± SEM. All dosage levels produced serum calcium and calcium transport values different from those in controls ($P < 0.01$–0.001). There were no significant differences among the 1α-OH-D_2, 1,25-$(OH)_2D_3$, and 1α-OH-D_3 groups within the same dose levels.

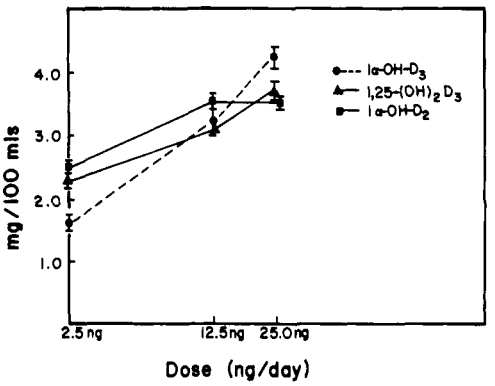

FIG. 3. Serum inorganic phosphorus concentration of vitamin D-deficient rats on a low phosphorus diet in response to the indicated daily doses of 1α-OH-D_3, 1,25-$(OH)_2D_3$, and 1α-OH-D_2. Weanling male rats obtained from the Holtzman Company were fed a 1.2% Ca, 0.1% P vitamin D-deficient diet for a period of 3 weeks at which time they were given daily interperitoneal injections of the indicated compounds dissolved in ethanol:propylene glycol (5:95) for a period of 7 days. The animals were killed and within 12 hr serum inorganic phosphorus levels were determined. The data are plotted as serum inorganic phosphorus levels (mg/100 ml) versus dose per day. Vertical bars are SEM for six rats. At 2.5 ng dose and 25 ng dose, 1α-OH-D_3 differs from 1α-OH-D_2 and 1,25-$(OH)_2D_3$, $P < 0.005$. There were no other significant differences.

in a mammalian species but appears to be considerably less toxic suggests that it may represent an important new compound for the treatment of metabolic bone disease.

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