THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 256, No. 2, Issue of January 25, pp. 823–826, 1981
Printed in U.S.A.

Synthesis and Biological Activity of Vitamin D_3-Sulfate*

(Received for publication, June 9, 1980)

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Vitamin D_3-3β-sulfate has been synthesized using pyridine sulfur trioxide as the sulfate donor. It has been shown to be pure by high performance liquid chromatography and spectral methods. Unlike previous reports, the product has been identified unambiguously as the 3β-sulfate ester of vitamin D_3 by its ultraviolet, nuclear magnetic resonance, infrared, and mass spectra. The biological activity of vitamin D_3-sulfate was then determined in vitamin D-deficient rats. Vitamin D_3-sulfate has less than 5% of the activity of vitamin D_3 to mobilize calcium from bone and approximately 1% of the ability of vitamin D_3 to stimulate calcium transport, elevate serum phosphorus, or support bone calcification. These results disprove previous claims that vitamin D_3-sulfate has potent biological activity, and they further do not support the contention that vitamin D-sulfate represents a potent water-soluble form of vitamin D in milk.

In recent years it has been suggested that conjugation of vitamin D with sulfate (SO_4) (Fig. 1) plays an important role in vitamin D metabolism. Higaki *et al.* (1) reported the *in vitro* formation of vitamin D_2-SO_4 by rat liver homogenates. They further suggested that vitamin D_2-SO_4 has approximately the same antirachitic potency as vitamin D_2 itself (2) but is less toxic when administered in large amounts (3). Unfortunately the authors failed to provide convincing evidence that the vitamin D_2-SO_4 had been prepared nor was satisfactory evidence of purity offered, casting doubt on the reported biological activity. Miravet *et al.* (4) repeated the experiments of Higaki using vitamin D_3, but they also failed to provide evidence of purity or physical evidence that the correct product was obtained. Thus, the reported potent biological activity of the vitamin D_3-3β-sulfate remained in considerable question.

The sulfate ester of vitamin D has also been isolated from the urine of both rabbits (1) and rats (5) after oral administration of massive amounts of the vitamin. High levels of a water-soluble form of vitamin D activity have been reported to occur in milk. Sahashi *et al.* (6) found cows' milk to contain 240 IU of vitamin D activity per liter, of which 80% was claimed to be water soluble. In addition, these authors report that human milk contains 965 IU of vitamin D activity per liter. Similar findings have been reported by Lakdawala and Widdowson (7). Both studies infer that this water-soluble form of vitamin D is the sulfate conjugate. Since it is generally believed that even fairly minor structural changes greatly affect the biological activity of analogs of vitamin D (8), it was of great importance to determine if vitamin D_3-SO_4[1] has biological activity and if it acts upon all of the target organs of vitamin D. The results of this study demonstrate that vitamin D_3-SO_4 has little or no biological activity, in contrast to previous reports.

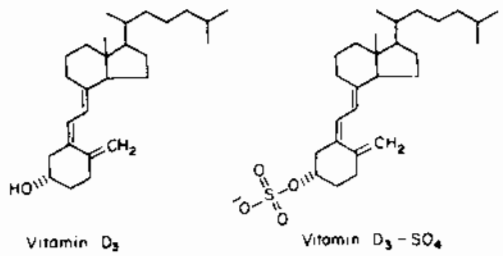

FIG. 1. **Structures of vitamin D_3 and vitamin D_3-SO_4.**

MATERIALS AND METHODS

Vitamin D_3 was purchased from the Thompson-Hayward Chemical Co., Kansas City, KS. Ultraviolet absorption spectra were recorded in HPLC grade methanol (Fisher Chemical Co., Chicago, IL) with a Beckman model 24 recording spectrophotometer. Nuclear magnetic resonance spectra were obtained with a Bruker 270-MHz spectrometer using CD_3OD as solvent; infrared absorption spectra were obtained with a Beckman model 4230. Mass spectrometry was performed with an AEI MS-9 mass spectrometer at 70 e.V. using a direct probe for introduction of the sample. HPLC was performed with a Waters model ALC/GPC 204 liquid chromatograph equipped with a Waters model 440 absorbance detector operating at 254 nm. A Partisil-10 ODS-2 semipreparative column (0.94 × 25 cm) (Whatman Co., Clifton, NJ) was used. Serum calcium concentrations were determined using a Perkin-Elmer model 402 atomic absorption spectrophotometer. Radioactivity (^{45}Ca) was measured by liquid scintillation counting with a Packard model 3255 counter. Samples were counted in a solution of toluene containing 0.2% 2,5-diphenyloxazole and 0.01% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene.

Synthesis of Vitamin D_3-SO_4—Vitamin D_3-SO_4 was synthesized according to a procedure similar to that of Sobel and Spoerri (9). Vitamin D_3 (1.0 g) and 1.0 g of pyridine sulfur trioxide were suspended in 6 ml of pyridine. Solution took place when the mixture was heated to 56°C; 0.4 ml of triethylamine was added and the reaction mixture held at 56–58°C for 20 min. At that time thin layer chromatography developed with methanol:$CHCl_3$ (1:4) indicated the disappearance of vitamin D_3 (R_F = 0.8) and the appearance of the product (R_F = 0.4). The solvent was removed by flash evaporation at a temperature of 40–45°C. The residue was dissolved in $CH_3OH/CHCl_3$ (1:9) and applied to a silica column (1.5 × 25 cm) eluted with the same solvent. A broad UV-absorbing peak eluting at approximately 5 column volumes was collected. A preliminary NMR spectrum identified it as the triethylamine salt of vitamin D_3-SO_4. In order to convert this product

* This work was supported by National Institutes of Health Program-Project Grant AM-14881 and the Harry Steenbock Research Fund of the Wisconsin Alumni Research Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: vitamin D_3-SO_4, vitamin D_3-3β-sulfate; HPLC, high performance liquid chromatography.

to the sodium salt, the solvent was evaporated and the residue dissolved in distilled water. Saturated NaCl was added dropwise until a white precipitate formed. The mixture was then centrifuged and the supernatant removed. The precipitate was redissolved in CH_3OH/$CHCl_3$ (1:9), applied to a silica column (1.5 × 25 cm), and eluted with the same solvent. A very broad UV-absorbing peak eluting at approximately 8 column volumes was collected. The solvent was evaporated, and the resulting compound was characterized by UV, NMR, and mass spectrometry. A second portion of the product was chromatographed on DEAE-Sephadex eluted first with methanol and then with methanol containing 0.4 M ammonium acetate. An additional portion of the compound was subjected to reversed phase HPLC, eluted with water:CH_3OH (1:4) containing 10 mM ammonium bicarbonate. It was then mixed with KBr, and an infrared spectrum was obtained.

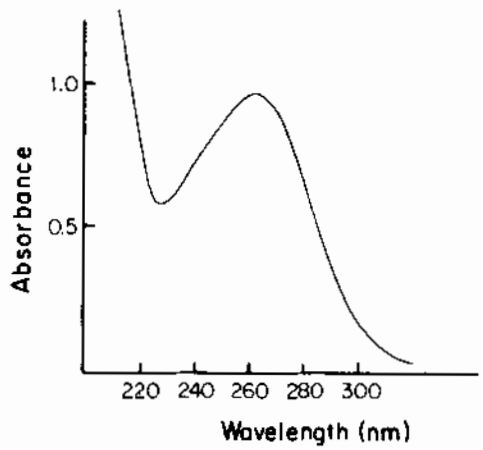

FIG. 2. **UV absorption spectrum of vitamin D_3-SO_4 dissolved in CH_3OH.** The maximum absorbance occurs at λ = 265 nm and the minimum at λ = 227 nm.

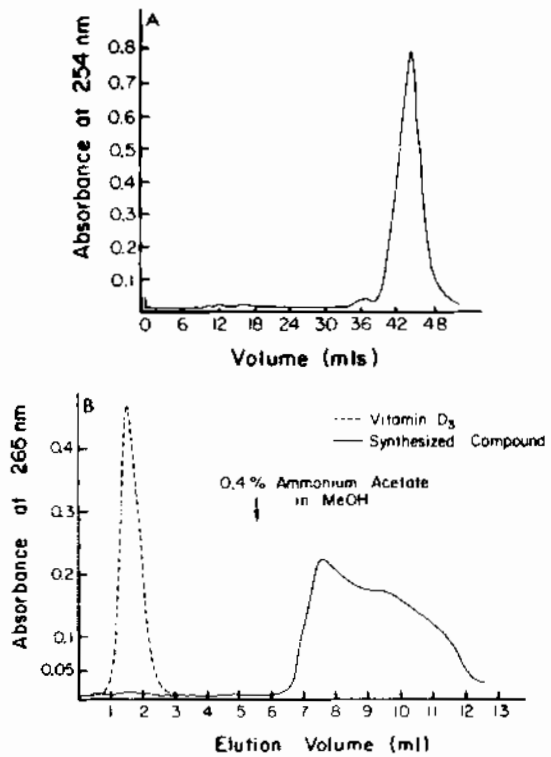

FIG. 3. **HPLC profile of vitamin D_3-sulfate.** *A*, reversed phase HPLC of vitamin D_3-SO_4 using an octadecylsilane column eluted with water:CH_3OH (1:4) containing 10 mM ammonium bicarbonate. Synthetic vitamin D_3-SO_4 migrated as a single peak at approximately 5 column volumes. *B*, chromatography of vitamin D_3-SO_4 on a DEAE-Sephadex column. – – –, elution of crystaline vitamin D_3 with CH_3OH. ——, synthesized vitamin D_3-SO_4 eluted with 0.4% ammonium acetate in CH_3OH.

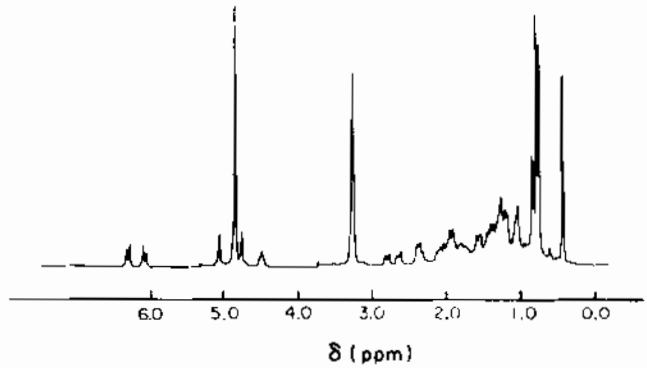

FIG. 4. **NMR spectrum of synthetic vitamin D_3-SO_4 dissolved in perdeutero-methanol (CD_3OD).** The peaks at δ 4.7 indicate an electronegative substituent on the 3β position. The prominent singlets at δ 3.2 and 4.9 are due to solvent.

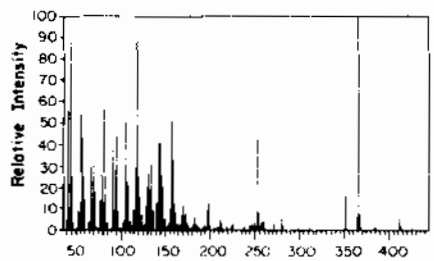

FIG. 5. **Mass spectrum of synthetic vitamin D_3-SO_4.** The base peak occurs at *m/e* 366 with additional peaks at *m/e* 253 and *m/e* 118.

Biological Assays—Weanling, male albino rats obtained from Holtzman Co., Madison, WI, were placed in individual hanging wire cages and were fed one of the vitamin D-deficient, purified diets previously described (10). The diets were either low calcium (0.02%), normal phosphorus (0.3%), or high calcium (1.2%), low phosphorus (0.1%). After a 3-week vitamin D-depletion period the rats were divided into groups of six animals. Each group of animals was given vitamin D_3 or vitamin D_3-SO_4 dissolved in 1,2-propanediol or the propanediol alone. The dosages were administered either intraperitoneally or orally for 7 consecutive days. Within 24 h after the last dose the rats were killed and their blood, duodena, distal ulnae, and radii were collected for the various determinations.

Intestinal Calcium Transport Assay—The rate of intestinal calcium transport in vitamin D-deficient, calcium-depleted rats was determined by the everted gut sac technique of Schachter and Rosen (11) as modified by Martin and DeLuca (12). The results are expressed as ratios of ^{45}Ca in the serosal medium to that in the mucosal medium.

Bone Calcium Mobilization—Blood obtained from the rats was centrifuged, and calcium determinations were carried out on 0.10 ml of serum diluted with 0.1% lanthanum chloride.

Determination of Serum Phosphorus—The serum inorganic phosphorus concentrations of rachitic rats were determined according to the procedure of Chen *et al.* (13).

Assay for Antirachitic Activity—The radii and ulnae were stained in 1.5% silver nitrate solution. New calcification on the epiphyseal plate was scored visually according to the U. S. Pharmacopoeia (14).

RESULTS

The 3β-sulfate ester of vitamin D_3 was produced under mild conditions using pyridine sulfur trioxide as the sulfate donor. The product of the reaction was converted to the sodium salt and purified by chromatography on a silica column. It was then eluted from reversed phase HPLC as a single peak at approximately 5 column volumes (Fig. 3A). The UV absorption spectrum of the reaction product indicates a maximum absorbance at λ = 265 nm and a minimum at λ = 227 nm (Fig. 2), which is also characteristic for vitamin D. However, vitamin D_3 is eluted from a DEAE-Sephadex column in approx-

imately 1 column volume of methanol (Fig. 3B). In contrast, the reaction product fails to elute from the same column in methanol and is eluted only with 0.4 M ammonium acetate in methanol. This indicates that the synthesized compound is charged and, therefore, cannot be vitamin D itself.

The NMR spectrum (Fig. 4) shows the typical pattern expected for vitamin D (15) except that the 1-proton multiplet which corresponds to the 3α-proton is shifted from δ 3.9 to 4.0 to δ 4.7. The spectrum confirms an unaltered *cis*-triene chromophore, and the shift of the 3α-proton is in the direction and magnitude expected for the conversion of hydroxy to an *O*-sulfate ester. Peaks at δ 3.0 and 1.3 which correspond to triethylamine are not present after the wash with saturated NaCl. This indicates that the Na salt of vitamin D-SO_4 has been formed.

The mass spectrum (Fig. 5) shows the expected elimination of the sulfate substituent to give the base peak at *m/e* 366 [M^+-(SO_4 + H] with additional fragments at *m/e* 253 (366-side chain) and *m/e* 118 (dehydro ring A plus carbon 6 and carbon 7). In contrast, the fragments characteristic of vitamin D_3 at *m/e* 384, *m/e* 271, and *m/e* 136 are very weak. Both the NMR and mass spectra are consistent with a vitamin D derivative containing an electronegative substituent on the 3β-hydroxyl position. The infrared spectrum of the synthesized compound (Fig. 6) contains absorption bands at 1240 and 1062 cm^{-1} which are not present in the infrared spectrum of vitamin D_3 itself (16) and may be compared to organic sulfate (17–19). From the physical data obtained, it is evident that the Na salt of vitamin D_3-3β-SO_4 has been synthesized.

Vitamin D_3-SO_4 exhibits very low activity in all of the classical assays for the functions of vitamin D. The data in Table I demonstrate that vitamin D_3-SO_4 retains less than 1% of the ability of vitamin D_3 to stimulate duodenal calcium transport. No stimulation of calcium mobilization from bone could be detected even when 6.6 nmol/day of vitamin D-SO_4 was administered (Table II). Vitamin D_3-SO_4 elevated the serum phosphorus concentration of phosphorus-depleted, vitamin D-deficient rats only after 6.5 nmol/day of the compound was administered (Table III). Approximately 1% of that amount of vitamin D itself causes a similar response. Calcification of newly formed bone could be detected only after 6.5 nmol/day of vitamin D_3-SO_4 was administered orally.

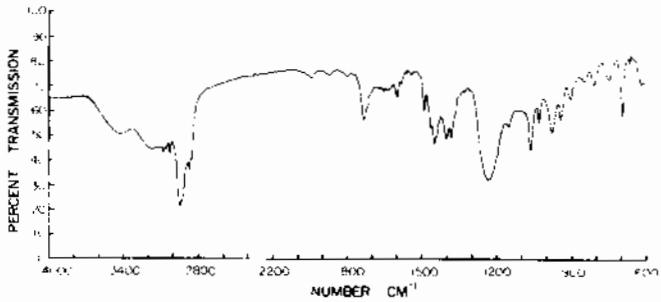

FIG. 6. **Infrared spectrum of vitamin D_3-SO_4.** Peaks at 1240 cm^{-1} and 1060 cm^{-1} are absent in the spectrum of vitamin D_3, and can be assigned to the sulfate ester function.

TABLE I

Effect of vitamin D-SO_4 on duodenal calcium transport

Rats which had received the vitamin D-deficient, low calcium diet for 3 weeks were divided into groups of six animals and dosed as indicated for 7 days. Calcium transport was measured by the everted duodenal sac method. Values are mean ± S.E.

Compound	Amount	Calcium transport
	nmol/day	*S/M ± S.E.*
Propylene glycol		2.1 ± 0.1
Vitamin D_3	0.026	7.2 ± 0.9
Vitamin D_3-SO_4	0.65	2.1 ± 0.2
	1.3	2.8 ± 0.3
	2.6	3.1 ± 0.2[a]
	2.6[b]	3.6 ± 0.6

[a] Differs from control at $P < 0.005$.
[b] Compound administered by intubation; all other groups were injected intraperitoneally.

TABLE II

Effect of vitamin D_3-SO_4 on bone calcium mobilization

Vitamin D-deficient, calcium depleted rats were dosed daily for 1 week. The data are expressed as the mean value of six animals ± S.E.

Compound	Amount	Serum calcium concentration
	nmol/day	*mg/100 ml ± S.E.*
Propylene glycol		4.0 ± 0.2
Vitamin D_3	0.33	6.0 ± 0.2
Vitamin D_3-SO_4	1.65	4.3 ± 0.1
	6.6	4.2 ± 0.2
	6.6[a]	4.6 ± 0.2[b]

[a] Compound administered by intubation; all others were injected intraperitoneally.
[b] Differs from control at $P < 0.05$.

TABLE III

Relative effectiveness of vitamin D_3-SO_4 on endochondrial calcification and maintenance of serum phosphorus concentration in rachitic rats

Groups of six vitamin D-deficient, phosphorus-depleted rats were given the indicated dose for 7 days. Serum phosphate concentrations are expressed as the mean ± S.E.

Compound	Amount	Serum phosphate	Calcification score
	nmol/day	*mg/100 ml ± S.E.*	
Propylene glycol		2.3 ± 0.4	0
Vitamin D_3	0.065	4.3 ± 0.1[a]	4–5
Vitamin D_3-SO_4	0.65	3.0 ± 0.5	0
	6.5	4.3 ± 0.3[a]	0
	6.5[b]	4.2 ± 0.3	2

[a] Differs from control at $P < 0.005$.
[b] Administered by intubation.

DISCUSSION

The biological activity of vitamin D_3 is reduced to less than 5% by conjugation of the 3β-hydroxyl with sulfate. This very low biological activity indicates that vitamin D_3-SO_4 has little or no activity itself. In addition, the sulfate ester is evidently stable under physiological conditions, and very little vitamin D is released *in vivo* from the substrate. This might be expected, since at room temperature vitamin D-SO_4 is stable in base and is only slowly hydrolized to release vitamin D in acid.[2] It is likely that vitamin D_3-SO_4 is hydrolyzed slowly in the stomach when the compound is administered orally. This would account for the slightly higher biological activity observed under these conditions.

The present results are in direct contrast to reports of high biological activity of vitamin D_2-SO_4 (3, 13) and vitamin D_3-SO_4 (4). In neither of those cases did the authors provide adequate evidence that the vitamin D-sulfate was obtained nor did they provide convincing evidence that the product was sufficiently pure for meaningful biological evaluation. As a result of those and other (1–7) similar reports there has been the widespread belief among nutritionists that vitamin D-sulfate has potent biological activity. The present study clearly dispels this belief.

The results of the present study also do not support the idea that vitamin D-sulfate is a potent form of vitamin D in

[2] L. LeVan and H. F. DeLuca, unpublished results.

milk (6, 7); if the sulfate-ester is in fact present in milk, it has little or no biological activity. Furthermore, the methods used in previous studies to assess the presence of vitamin D-sulfate in milk are questionable, making the results difficult to evaluate. This question must, therefore, be re-examined.

The question of whether vitamin D-sulfate is an important excretory form of the vitamin has not been addressed in the present study. The significance of earlier reports of vitamin D_3-SO_4 as an excretory product in animals (1, 4) is difficult to assess, in view of the massive amounts of vitamin D_3 administered in these studies and the very low abundance of sulfate ester recovered. This question should be examined more closely before its role as an excretory product can be assessed. At present, however, the concept of a highly biologically active vitamin D-sulfate can be discarded.

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