Synthesis and Biological Activity of Vitamin D₃ 3β-Sulfate

ROLE OF VITAMIN D₃ SULFATES IN CALCIUM HOMEOSTASIS*

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To determine the biological activity of vitamin D sulfates, we synthesized vitamin D₃ 3β-sulfate and tested its biological activity in vitamin D-deficient hypocalcemic rats. When vitamin D₃ sulfate was administered as a single oral dose of 208,000 or 416,000 pmol (100 µg or 200 µg), it increased active calcium transport in the duodenum and was also able to mobilize calcium from bone and soft tissue. Dose levels below this failed to elicit a response. Vitamin D₃ sulfate itself was active at doses as low as 260 pmol when administered in this manner. In order to test the biological activity of vitamin D₃ sulfate in various doses when administered chronically, we tested the biological activity of vitamin D₃ sulfate after 5 days of oral dosing: vitamin D₃ sulfate was active at doses of 52,000 pmol/day (25 µg), whereas vitamin D₃ was active at doses of 65 to 260 pmol/day over a period of 5 days. When administered as a single intravenous dose, vitamin D₃ sulfate exhibited no biological activity in doses as high as 52,000 pmol. Vitamin D₃, however, was active at a dose of as low as 65 pmol. We conclude that vitamin D₃ sulfate, a metabolite of vitamin D₃ of heretofore unknown biological activity, is considerably less active than vitamin D₃ itself.

The role of vitamin D₃ sulfate¹ in calcium homeostasis is unclear. Vitamin D₃ sulfate has been isolated from rabbit urine, rat liver homogenates, and chicken tissues (Higaki et al., 1965); human and cow's milk (Sahashi et al., 1967) may also contain vitamin D₃ sulfate. Additionally, "vitamin D sulfate" has been isolated from milk (Lakdawala and Widdowson, 1977), although precise chemical identification and biological potency testing was not performed. Vitamin D₃ sulfate is biologically active in rats, and when vitamin D₃ sulfate (10 IU or ~250 ng) was administered for 28 days to rachitic rats, it was effective in maintaining growth, increasing the mineral ash content of bones, and healing rickets as assessed by x-ray analysis of the bones (Sahashi et al., 1969). The antirachitic activity of human milk due to free vitamin D or nonconjugated vitamin D metabolite activity has been estimated (Lakdawala and Widdowson, 1977) to be between 0.01 and 0.15 µg/dl, a value below the amount required to prevent rickets in a normal human infant. Therefore, it has been postulated that because breast-fed infants do not become rachitic, the antirachitic activity of milk is probably due to the biological activity of vitamin D sulfates. It has been hypothesized that the hydrolysis of vitamin D sulfate to free vitamin D could result in the formation of sufficient vitamin D to prevent rickets. Various workers have claimed that human milk contains between 1 to 2 µg/dl of putative vitamin D sulfate (Lakdawala and Widdowson, 1977). Extensive data concerning the biological activity of vitamin D₃ sulfate are not available. Sorgue and Miravet (1978) synthesized [³H]vitamin D₃ sulfate and administered it to young rats. No free D₃ was isolated 7 h after administration. Le Boulch et al. (1979, a and b) reported that in the lactating and gestating female rat, vitamin D₃ sulfate was hydrolyzed to free "vitamin D₃." However, vitamin D₃ was not identified by any method other than chromatographic mobility on LH-20 Sephadex. Marnay-Gulat et al. (1975) showed that vitamin D₃ sulfoconjugate was inactive in chickens. Miravet et al. (1975) showed that vitamin D₃ sulfate had some biological activity in young rats, although only two dose ranges were tested in small numbers of rats. To clarify these ambiguities, we synthesized vitamin D₃ 3β-sulfate in pure form (judged by ultraviolet spectroscopy, mass spectroscopy, elemental analysis, nuclear magnetic resonance spectroscopy, and liquid chromatography) and tested its biological activity in vivo.

MATERIALS AND METHODS

All ultraviolet absorption spectra were taken in ethanol on a Beckman model 25 (Beckman Instruments, Palo Alto, CA) recording spectrophotometer. All mass spectra were recorded on Hewlett-Packard spectrometer model 5985A (Hewlett-Packard, Avondale, PA) equipped with a direct insertion probe. All NMR spectra were recorded on an IBM 80 MH, NMR spectrometer. Infrared spectra were recorded on a Perkin-Elmer 327 infrared spectrometer. Serum calcium was determined with an atomic absorption spectrometer (Waters Associates, Milford, MA) equipped with a variable UV detector and a gradient mixer. All melting points were uncorrected. Elemental analysis was performed by Galbraith Laboratories, Knoxville, TN.

High performance liquid chromatography was performed using a Waters LC 204 liquid chromatograph (Waters Associates, Milford, MA) equipped with a variable UV detector and a gradient mixer. All melting points were uncorrected. Elemental analysis was performed by Galbraith Laboratories, Knoxville, TN.

Synthesis of 3β-Sulfoxy-9,10-secocholesta-5,7,10(19)-triene Sodium Salt—Pulverized sulfamic acid (20 mg, 0.2 mmol) was suspended in freshly distilled dry pyridine (2 ml) and vitamin D₃ (38.4 mg, 0.1 mmol) was added to the suspension (Higaki et al., 1965). The mixture was vigorously stirred at 90-95 °C (bath temperature) for 1 h. Once the reaction was complete (as judged by thin layer chromatography), the pyridine-sulfamic acid mixture was filtered and the solvent was removed on a rotary evaporator. The residue was stirred with ether (3 ml) and was kept at 0 °C overnight. The solid obtained in this manner was chromatographed on a Silica Gel 60 H column, with an eluent of 100% ethyl acetate to 30% methanol/ethyl acetate.

Vitamin

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The abbreviations used are: vitamin D₃ sulfate, 3β-sulfoxy-9,10-secocholesta-5,6,10(19)-triene sodium salt; vitamin D₃ sulfate, 3β-sulfoxy-9,10-secoergosta-5,7,10(19)-triene ammonium salt; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; IR, infrared.
D$_3$ sulfate (yield 85%) ammonium salt obtained as colorless crystals (m.p. 105.5-107°C)

The vitamin D$_3$ sulfate ammonium salt (100 mg) was dissolved in pyridine (2 ml) and a 15% aqueous solution of sodium hydroxide (3 ml) was added (Joseph et al., 1966). The reaction mixture was stirred at room temperature for 3 h. The organic phase was separated and extracted with ether (3 x 5 ml). The combined organic layers were dried over anhydrous sodium sulfate. Solvent was evaporated to obtain a faintly solid (yield 80%) which was further purified on silica Gel 60 H (eluant: ethyl acetate to 30% methanol in ethyl acetate).

The vitamin D$_3$ sulfate salt thus purified was further purified by HPLC on a reverse phase, C$_{18}$Bondapak column using a linear gradient from 35% acetonitrile in water to 100% acetonitrile solvent system. Flow rate was 4 ml/min and the gradient was developed over a period of 18 min. The vitamin D$_3$ sulfate eluted at 4.75 min, and a minor amount (less than 3%) of vitamin D$_2$ was eluted at 16.31 min. The sulfoconjugate fraction was collected and solvent was removed to obtain colorless pure compound (m.p. 126-128°C). Ultra violet spectroscopy showed $\lambda_{max}$ at 265 nm and $\lambda_{max}$ at 228 nm. IR (KBr) 1175-1275 (va), 1700 (s) and 1035 (m) (asymmetric and symmetric S=O cm$^{-1}$. NMR (CDCl$_3$) 6 6.15 (AB quartet, H-6,7; J = 12 HZ), 5.03 (broad s, H-19), 4.86 (broad s, H-19), 4.65 (broad s, H-3), 0.55 (s, H-C-18).

The molecular ion peak was not recorded in the mass spectrum but peaks at m/z 367 (M$^+$ - SO$_4$Na), m/z 366 (M$^+$ - HSO$_4$Na) were recorded. Prominent peaks were observed for SO$_4$$^-$ (m/z 48) and SO$_3$$^-$ (m/z 64).

$$\text{C}_x\text{H}_y\text{O}_z\text{Na}_a\cdot\text{H}_2\text{O}$$

Calculated: C 65.41 H 8.95 S 6.46

Found: C 65.08 H 8.88 S 6.38

Animals—Fifty to sixty-g, weanling, albino, male rats were obtained from the Holtzman Co. (Holtzman C6, Madison, WI). They were maintained in individual overhanging wire cages in UV free light and were fed ad libitum a vitamin D$_2$-deficient, 0.2% calcium, 0.3% phosphorus diet. After 4 weeks on this diet, the animals were used for the experiments described below.

Experimental Design—The doses of vitamin D$_3$ sulfate were purified by high performance liquid chromatography immediately before use. No free vitamin D$_3$ was present in this preparation. In the first experiment, a group of rats received varying doses of vitamin D$_3$ sulfate dissolved in 33% aqueous ethanol (65 to 52,000 pmol) intravenously. Blood (500 to 500 ml) was obtained prior to the administration of the dose. Another group of animals received the corresponding doses of vitamin D$_3$ dissolved in ethanol orally. The doses were administered by intubation directly into the stomach.

In a third experiment designed to test the biological activity of the vitamin D$_3$ sulfate when administered chronically, a group of rats received varying doses of vitamin D$_3$ sulfate orally (65 to 52,000 pmol) every day for 5 days. For purposes of comparison, another group of rats received vitamin D$_3$ in the same dose for the same period of time.

There were six to nine animals in a group at each dose tested. Control groups had 12 to 15 animals/group. The values reported are the results mean ± S.E. of each such group of animals.

Measurement of Serum 25-Hydroxyvitamin D$_3$—This was measured using a modified method with high pressure liquid chromatography (Eisman et al., 1977). After collecting the appropriate fractions from the high performance liquid chromatography column, all samples that contained low or undetectable (by UV) 25-hydroxyvitamin D$_3$ concentrations were assayed by a competitive binding assay.

Statistical Analysis—This was performed using the Student's t test.

**RESULTS**

Analysis of vitamin D$_3$ sulfate by mass spectroscopy revealed a fragmentation pattern compatible with the proposed structure. IR spectroscopy showed the presence of S=O stretch. NMR spectroscopy demonstrated the change in the chemical shift of the 3α proton induced by the presence of a sulfate moiety. UV spectroscopy demonstrated absorption characteristic of a 5,6-cis-triene of vitamin D. HPLC demonstrated a single polar peak. The hygrosopic vitamin D$_3$ sulfate appears to have crystallized with half a mole of water as shown by elemental analysis. Dehydration above room temperature was avoided as the compound would decompose with the elimination of the sulfate group. All these facts would suggest that we have synthesized the appropriate compound in the form pure.

Fig. 1A demonstrates the active duodenal transport responses following the intravenous administration of vitamin D$_3$ or vitamin D$_3$ sulfate to vitamin D$_3$-deficient rats raised on a low calcium diet. It is clear that vitamin D$_3$ is active at doses as low as 65 pmol. However, at doses of up to 52,000 pmol, vitamin D$_3$ sulfate was inactive. In Fig. 1B are shown the changes in serum calcium when vitamin D$_3$ or vitamin D$_3$ sulfate was administered to vitamin D-deficient rats. The change in serum calcium (milligrams per dl) is plotted against the dose administered. At doses above 280 pmol, vitamin D$_3$ is effective in mobilizing bone and soft tissue.

**FIG. 1.** Comparison of biological activity of intravenous doses of either vitamin D$_3$ or vitamin D$_3$ sulfate. A, active duodenal transport of calcium in vitamin D-deficient rats administered either vitamin D$_3$ (○) or vitamin D$_3$ sulfate (▲) intravenously 24 h before the experiment. Values for the control groups are shown on the ordinate. There were six to nine rats at each dose tested. *p < 0.05, **p < 0.02, ***p < 0.005, ****p < 0.001. B, change in serum calcium levels (Δ Ca mg/dl) in vitamin D$_3$-deficient rats administered either vitamin D$_3$ (○) or vitamin D$_3$ sulfate (▲) intravenously 24 h before the experiment. Values for the control groups are shown on the ordinate. There were six to nine rats at each dose. *p < 0.005, **p < 0.001. Values for the Δ Ca for vitamin D$_3$ sulfate at doses greater than 1,000 pmol extend along the abscissa.
calcium. Vitamin D$_3$ sulfate is inactive in this respect at doses as high as 52,000 pmol. In Fig. 2A are shown the results of the active calcium transport in the duodenum following a single oral dose of vitamin D$_3$ or vitamin D$_2$ sulfate. Vitamin D$_2$ is active at a dose of 260 pmol, whereas vitamin D$_3$ sulfate exhibits activity at 208,000 pmol and higher. In Fig. 2B are shown the results of the change in serum calcium following a single oral dose of vitamin D$_3$ or vitamin D$_2$ sulfate. Vitamin D$_3$ sulfate is active at doses greater than 208,000 pmol. When vitamin D$_3$ sulfate or vitamin D$_2$ were administered orally, once per day, for a period of 5 days, there was an increase in active calcium transport at doses of 52,000 pmol or higher in the case of vitamin D$_2$ sulfate; vitamin D$_3$ itself was active at a dose of 65 pmol/day (Fig. 3A). Vitamin D$_3$ sulfate mobilized bone/soft tissue calcium at doses of 104,000 pmol when administered chronically; vitamin D$_2$ was active at doses as low as 260 pmol (Fig. 3B). The results of increments in weight and serum phosphorus following oral dosing with vitamin D$_3$ or vitamin D$_2$ sulfate are shown in Table I. It is clear that serum phosphorus and weight increases at the highest doses of D$_3$ sulfate used, whereas vitamin D$_2$ is effective at low doses.

The results of serum 25-hydroxyvitamin D$_3$ levels at the various doses of vitamin D$_3$ or vitamin D$_3$ sulfate administered...
as an oral dose are shown in Table II. It is clear that 25-hydroxyvitamin D$_3$ is present in the serum of rats administered vitamin D$_3$ at a dose of 1,040 pmol or more. At a dose of 52,000 pmol/day, vitamin D$_3$ sulfate exhibits no significant increment in serum 25-hydroxyvitamin D$_3$ levels.

### Discussion

Conjugates of vitamin D or its analogs are known to occur in rat bile (Kumar et al., 1980), and more recently, we have demonstrated that one of these can be reutilized (Nagubandi et al., 1980). We report here the biological activity of another conjugate, vitamin D$_3$ sulfate, in young rats. When administered intravenously, vitamin D$_3$ sulfate is not active at the doses tested. This could be due to rapid clearance by renal tubular transport processes. Alternatively, the sulfate could be inactive at the doses we administered. When administered as a single oral dose, vitamin D$_3$ sulfate is active at doses in excess of 50,000 pmol. When administered over a period of 5 days, vitamin D$_3$ sulfate is active at doses of 52,000 pmol/day. Therefore, chronic oral dosing is an effective method for eliciting a response to the sulfate. Our data do not allow us to determine whether vitamin D$_3$ sulfate is active as an intact moiety, or only after hydrolysis to vitamin D$_3$. At the highest doses of vitamin D$_3$ sulfate administered, there were small but statistically significant changes in serum 25-hydroxyvitamin D$_3$ levels. Lower doses of vitamin D$_3$ (65 to 520 pmol/rat/day for 5 days) showed biological activity but failed to raise the serum 25-dihydroxyvitamin D$_3$ levels to a significant extent when assessed by our method. Experiments with radiolabeled D$_3$ administered to rachitic animals show the presence of radiolabeled 25-hydroxyvitamin D$_3$ in blood, suggesting that our methods may be too insensitive to detect small changes in serum 25-hydroxyvitamin D$_3$. Earlier work by Sahashi et al. (1969) demonstrated that vitamin D$_3$ sulfate was a potent antirachitic agent, when administered in a dose of 10 IU (~250 ng) for a period of 28 days. Further, it appeared that vitamin D$_3$ sulfate was nearly as active as vitamin D$_3$ itself. Marnay-Gulat et al. (1975) showed that chronic oral administration of vitamin D$_3$ sulfoconjugate to vitamin D-deficient chickens had little effect on growth, serum calcium levels, or healing of rickets. Miravet et al. (1975) showed that vitamin D$_3$ sulfoconjugate was active in young vitamin D-deficient rats administered this analog of vitamin D. In these experiments, the sulfate of vitamin D$_3$ was tested at two dose levels only. The duodenal calcium transport ratios (mucosal-serosal) were extremely low even in the vitamin D$_3$- or 1,25-dihydroxyvitamin D$_3$-treated group, raising the possibility of technical problems in that particular experiment. Similarly “bone calcium mobilization” was tested at one dose level only in rats raised on a normal calcium diet (0.47% calcium). We feel that the presence of normal calcium concentrations in the diet makes it difficult to state, unequivocally, the source of calcium that results in the serum calcium increments. We cannot account for the differences in the activity observed by us relative to other investigators.

In any event, our results using biological tests of vitamin D$_3$ function lead us to believe that young rats can utilize vitamin D$_3$ sulfate when it is administered chronically via the oral route at doses of 52,000 pmol or higher. Whether vitamin D$_3$ sulfates acts without being hydrolyzed to vitamin D$_3$ or acts after hydrolysis to free D$_3$ is not very clear. We conclude that the vitamin D$_3$ sulfate is biologically active only in high doses when administered orally to young rats. It is possible that vitamin D$_3$ sulfate could be utilized by infants as a source of vitamin D. Whether or not the amounts present in milk can be utilized efficiently by the human infant remains unclear.

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### Table II

| Compound administered | Dose administered | 25(OH)D$_3$ pmol ng/dl (mean ± S.E.) |
|------------------------|-------------------|-----------------------------------|
| Ethanol                |                   | 0.91 ± 0.24                       |
| Vitamin D$_3$          | 65                 | 1.55 ± 0.05*                      |
|                        | 260                | 1.83 ± 0.15*                      |
|                        | 520                | 2.15 ± 0.31*                      |
|                        | 1,040              | 3.27 ± 0.54                       |
|                        | 2,080              | 3.47 ± 0.29                       |
|                        | 52,000             | 39.57 ± 3.50                      |
|                        | 104,000            | 74.67 ± 1.67                      |
|                        | 208,000*           | 73.8 ± 4.60                      |
|                        | 416,000*           | 131.6 ± 4.38                     |
| Vitamin D$_3$ sulfate  | 52,000             | 0.88 ± 0.57*                      |
|                        | 104,000            | 1.15 ± 0.29*                      |
|                        | 208,000*           | 2.53 ± 0.32*                      |
|                        | 416,000*           | 2.23 ± 0.14*                      |

* p not significant compared to controls (p > 0.05).

* p < 0.05 compared to controls.