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Lack of biological activity of vitamin D_3-3β sulfate during lactation in vitamin D-deficient rats

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Summary. Sulfoconjugated vitamin D has been claimed to have an important antirachitic activity and to be present at higher amounts than free vitamin D in maternal milk. We have previously shown that vitamin D_3-3β sulfate (SD_3) administration has little effect on calcium and bone metabolism during pregnancy in rats. In the present work, we have compared the biological activity of free vitamin D_3 (D_3) and SD_3 during the lactation period. After delivery, D-depleted (-D) female rats were orally treated with D_3 or SD_3 (1 300 pmoles/every two days) during 20 days of lactation. Vitamin D status was determined before, during and at the end of the treatment for mother rats and at days 1 and 20 of life in suckling pups. Both mothers and pups were sacrificed at day 20 of lactation and subjected to hormonal and mineral determinations and to histomorphometric analysis of bone metabolism. After 12 days of SD_3 treatment, mother rats showed a slight but significant elevation in plasma concentrations of calcium phosphorus and vitamin D metabolites. This effect was reversed at the end of lactation ; at this time most maternal plasma parameters did not differ from those observed in -D non-treated mothers. By contrast, 20 days of D_3 administration in mothers normalized plasma biochemical parameters. These results were confirmed by analysis of both static and dynamic parameters of bone formation. Maternal SD_3 treatment did not improve either plasma biochemical or histological parameters of bone formation and resorption in suckling pups which remained comparable to that of D-deficient pups ; by contrast, pups from D_3-treated mothers normalized most biochemical plasma parameters although bone metabolism remained abnormal. In conclusion, the biological activity of SD_3 on bone and mineral metabolism during lactation in rats is as low as in the non-reproductive stages.

Introduction.

Mother's milk is the major source of vitamin D during the first weeks of life in mammals. However, the vitamin D content of milk has been reported to be low (15-40 IU/liter) (1, 2, 3). Normal growth rates in suckling newborns have been suggested to be due to the presence in maternal milk of a water-soluble form of vitamin D identified as vitamin D sulfate (4). The presence of large amounts of vitamin D sulfate in milk has been initially confirmed (5-10) but was denied later on (2, 11-13). At the same time, initial studies have reported that this vitamin compound may have some biological activity (14-16). Thus in vitamin D-deficient

male rats vitamin D sulfate is almost as active as free vitamin D on bone development, as evaluated by the « line » test and bone ashes (14). By contrast, other investigators have been unable to find any difference in the antirachitic potency of either whole milk or its lipid fraction (11). Also, Reeve *et al.* (2) have not found significant differences in intestinal calcium transfer in the male rat after either treatment with 25 ng of vitamin D_3 (= 1 IU)/day or oral administration corresponding to 25 ml of whole human milk. Furthermore, those authors observed that human milk contained 40 IU of D activity/liter, mainly due to vitamin D and its mono- and dihydroxylated metabolites. From these results and other studies (17-19), the presence of a highly potent water-soluble form of vitamin D in maternal milk was unlikely. However, Le Boulch *et al.* have observed in the rat both « in vivo » hydrolysis (20) and « in vivo » synthesis (21) of radiolabelled vitamin D_3 sulfate during pregnancy and at the first stages of lactation. Therefore, it seemed necessary to extend previous studies to the pregnant and lactating stages.

Recently we have shown that chronic administration of synthetic vitamin D_3-3β sulfate (SD_3) to the female rat, before and during pregnancy, had less biological activity than an equal administration of free vitamin D_3 (22). However, since vitamin D sulfate may be present in milk, it is still possible that this vitamin D may play an important role during lactation, particularly in suckling newborns. The present study was designed to compare the biological activity of both free and sulfoconjugated synthetic vitamin D_3 in mother rats and their litters during lactation.

Material and methods.

Material. — Vitamin D_3 was obtained in a crystalized form from Philip Duphar (The Netherlands). The 25-hydroxyvitamin D_3 (25 OH D) was kindly supplied by Roussel Laboratory, France. The 24R,25-dihydroxyvitamin D_3 (24, 25$(OH)_2D_3$) and 1α,25 dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) were supplied by Hoffman-LaRoche Laboratories.

Sulfoconjugated vitamin D_3 was synthesized in our laboratory as sodium salt according to a previously described method (23). The purity of the compound was determined by high-pressure liquid chromatography (HPLC). It contained less that 1/1 000 of free vitamin D_3. The identification of the SD_3 was verified both by ultra-violet spectrometry (λ max = 265 nm, λ min = 228 nm, characteristic of the 5-6 cis triene conformation) and mass spectrometry (methodology : Desorption Chemical Ionisation ; reagent gas used = N_{48} ; Typical ions observed : mz 366 = base peak, resulting from loss of HNa SO_4 ; mz 351 and mz 253 resulting respectively from loss of methyl and side chain C_8H_{17} of ion 366) as reported elsewhere (10).

All reagents used were of « high purity » grade and all solvents were of « chromatography » grade. Prior to HPLC use, all solvents were filtered over a 0.4-μm membrane (Schleicher and Schull, West-Germany) and gas-freed.

Radiolabelled vitamin D_3 metabolites, as well as ^{125}ICl, with a specific activity of 2 960 to 4 070 GBq/mmol, were purchased from Amersham France.

Synthetic human PTH and antiserum were kindly supplied by Dr M. Moukhtar (INSERM U1113, Hôpital St Antoine, Paris, France).

Animals and diets. — Female rats of the Cobs strain (Charles River France) were obtained at 3 weeks of age and placed on a vitamin D-free diet (I.N.R.A., La Minière, France) containing 0.45 % Ca and 0.30 % P, vitamin D-free casein (18 %), corn starch (10 %) and cerelose (64.5 %) as the major energy sources. The rats were housed separately in polystyrene cages, kept away from UV light and allowed free access to food and deionized water.

Experimental protocol. — The experiment was carried out over 14 weeks after weaning. Vitamin D-replete animals (+ D) were orally supplemented with 1 300 pmoles of vitamin D_3 every two days from weaning to sacrifice. In order to allow normal mating and pregnancy vitamin D-deficient animals received a minimal amount of vitamin D_3 (130 pmoles/week, orally) between weeks 4 and 8 of the experiment. All female rats were mated at 11 weeks of age (= after 8 weeks of experiment) with age-matched + D male rats from the same strain. After delivery, one group of D-deficient mothers was kept untreated (− D ; n = 6), and two other groups were either supplemented with free vitamin D_3 (− D + D, n = 5) or with equal amounts of sulfoconjugated vitamin D_3 (− D + SD, n = 6). Litters were reduced to five pups on day 1 after birth. Both D_3 and SD_3 supplementations were orally administered after dilution in 0.1 ml of neutralized vitamin D-free olive oil.

Blood and bone samples. — Vitamin D was determined in blood samples obtained from the tail vein at 4 and 8 weeks after weaning and at day 18 of pregnancy (P18).

At days 1 (L1), 12 (L12) and 20 (L20) of lactation, the mothers were bled under mild ether anaesthesia by tail section (L1 and L12) or heart puncture (L20). Blood was collected in heparinized tubes. Newborn pups were sacrificed at day 1 of life. Blood was collected under ether anaesthesia using an heparinized Pasteur pipette after section of the brachial vessels. Twenty day-old pups were bled by heart puncture and samples were collected using heparinized syringes under mild ether anaesthesia.

The plasma samples were immediately analyzed for their Ca, Mg and P contents. The remaining plasma samples were frozen and kept at − 20 °C until required for hormonal and vitamin D metabolite determinations.

At autopsy, to compare bone growth among the different groups, femur length was determined in both mothers and 20-day old pups. Also, the right femur of the mothers, the right femur-tibia fibula of 20-day old pups and whole new born pups were collected for determination of their mineral contents. Caudal vertebrae from mothers (7th) and 20-day old pups (7th and 8th) were cleaned of soft tissue and prepared for quantitative histomorphometric determinations.

Plasma biochemical determinations. — Plasma samples were diluted in a 5 % lanthanum chloride spectral solution and analyzed for total calcium (Ca) and

magnesium (Mg) content by atomic absorption spectrometry (Perkin-Elmer 303). Plasma phosphate was determined as inorganic phosphorus by the method of Chen, Toribara and Warner (24).

Except for newborn pups, plasma levels of 25(OH)D, $24,25(OH)_2D$ and $1,25(OH)_2D$ were determined in both mothers and pups in individual samples of 0.5 – 2 ml. Extraction and purification of vitamin D metabolites were performed using pre-established methods including ammoniac-protein precipitation extraction (25), silica Sep-Pak (Waters) separation (26) and high-pressure liquid chromatography purification (27). Overall recoveries were 80-95 % for 25(OH)D and 65-80 % for $24,25(OH)_2D$; both metabolites were measured by a competitive protein-binding assay using normal rat kidney cytosol as ligand (28). Sensitivity from the standard curve ranged from 80 to 100 pg/assay tube and intra and interassay variability did not exceed 10 %. Overall recovery for $1,25(OH)_2D$ was 80-95 % and its concentration was determined by a competitive binding assay which uses normal chick intestinal cytosol as the protein source (27). Assay sensitivity ranged from 1.5 to 3 pg/assay tube and variability did not exceed 15 %. Non-specific binding was determined for each metabolite in individual plasma samples by adding a 200-fold excess of the unlabelled metabolite assayed. Samples were assayed in triplicate.

Plasma concentrations of immunoreactive (i) parathormone (PTH) were measured by radio-immunoassay (RIA). Synthetic human PTH (1-34) was used as a standard and labelled with ^{125}I following the method of Hunter and Greenwood (29). Antiserum was used at a final dilution of 1 : 200 000 and the separation of bound from free hormone was accomplished with dextran-coated charcoal. The lower limit of detectability of the assay was 15 pg of iPTH/ml. Samples were assayed in duplicate and non-specific binding determined for each sample. Intra and interassay variations did not exceed 15 %. The assay for iPTH was validated by the use of plasma from surgically hypoparathyroid rats. The antiserum used has been shown to cross-react with rat PTH (30). In this study, the levels measured in + D control groups were considered to be and were used as normal concentrations.

Bone ash determination. — Bone specimens, cleaned of soft tissues, dehydrated and defatted by successive passages in ethanol, diethylether and acetone, were lyophylized prior to determination of dry weight. After ashing at 600 °C for 12 h, bone ashes were weighed and dissolved in 6 N HCl. Total bone mineral content was determined using the methodology already described for plasma samples.

Quantitative histology. — Skeletal changes were evaluated by quantitative histology. Mother rats were given an intraperitoneal injection of tetracycline (Terramicin, Pfizer Laboratories, Orsay-France ; 30 mg/kg body wt), 8 and 4 days before sacrifice. The caudal vertebrae were fixed in cold phosphate-buffered 10 % formalin (pH 7.2) and then dehydrated in ethanol before being embedded without decalcification in methyl methacrylate. Longitudinal sections were cut at 5-μm thickness using a Jung model K motorized microtome and stained with toluidine blue for evaluation of static variables ; 15-μm unstained sections were mounted for evaluation of double tetracycline incorporation into bone.

Measurements were performed on the endosteal area of the vertebrae, using previously described histomorphometric methods (22, 31). The following indices of bone formation and resorption were measured : osteoid volume (VO : % of bone tissue composed of uncalcified bone matrix), osteoid surface (% of endosteal surface covered by an osteoid seam), osteoblastic surface (% of endosteal surface showing plump osteoblasts), mean osteoid seam thickness, osteoclastic surface (Ost. S. : % of endosteal surface showing osteoclasts in resorptive lacunae), number of osteoclasts per mm^2 of bone tissue and calcified bone volume (CBV : % of total bone tissue). In mother rats double tetracycline labelling allowed measurement of the calcification rate (mean distance between the two labels divided by the time interval between the two injections), double-labelled surface (DL : % of endosteal surface exhibiting a double fluorescent tetracycline label), and endosteal mineralization lag-time (MLT : ratio of the mean osteoid thickness over the calcification rate). In pups, the status of epiphyseal mineralization was evaluated by measuring the mean thickness of the hypertrophic zone of the growth plate.

Statistical analysis. — All results were expressed as mean ± SEM. Differences between groups were determined using a one-way analysis of variance and Barlett's test.

Results.

A. — *Plasma biochemical parameters*

Effect of vitamin D depletion

Normal breeding in − D female rats was achieved by giving small amounts of vitamin D_3 during the 4 weeks preceding mating. After 18 days of D-deficient pregnancy (P_{18}), plasma levels of 25(OH)D were nearly undetectable in plasma of − D pregnant rats compared to values in + D pregnant rats (− D : 1.0 ± 0.4 *vs* + D : 10.3 ± 0.4 ng/ml ; $p < 0.001$). However, plasma levels of Ca and P were only slightly decreased in − D as compared to those of + D pregnant rats (plasma Ca : − D = 10.3 ± 0.1 *vs* + D = 11.4 ± 0.1 mg/dl, $p < 0.001$; plasma P : − D = 6.8 ± 0.2 *vs* + D = 7.2 ± 0.2 mg/dl ; $p < 0.05$).

One day after delivery (L_1) vitamin D-deficient mothers showed a marked decrease in plasma levels of Ca and P when compared to concentrations measured at P_{18} (fig. 1). At that time, plasma levels of 25(OH)D were undetectable (< 1 ng/ml) in − D mothers (fig. 2). In vitamin D-repleted mothers, the plasma levels of Ca and P, though within normal range, showed a similar trend (fig. 1) ; the 25(OH)D concentration in maternal plasma did not change significantly between P_{18} and L_1 (fig. 2).

Vitamin D-deficiency between L_1 and L_{20} in maternal rats led to a marked fall in plasma levels of Ca and P (fig. 1), whereas plasma 25(OH)D and $24,25(OH)_2D$

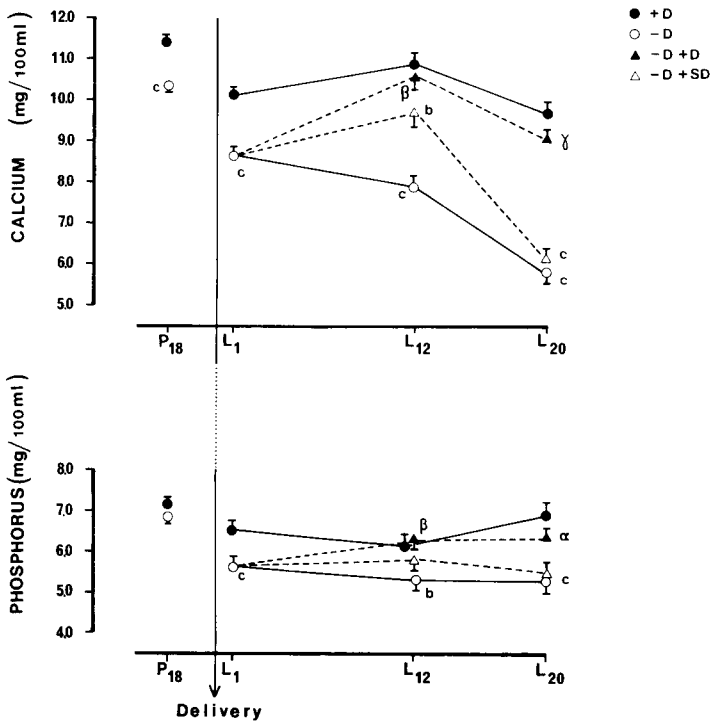

FIG. 1. — *Maternal plasma levels of calcium and phosphorus measured in controls* (+ D = D-repleted ; − D = D-depleted) *and treated rats* (− D + D and − D + SD : − D mothers receiving D_3 and SD_3, respectively, during lactation). P_{18} = day 18 of pregnancy L_1, L_{12}, L_{20} = day 1, 12 and 20 of lactation respectively.

Results are expressed as mean ± SEM of 5-6 individual determinations. Statistical differences were determined by analysis of variance and Barlett's test.

b = $p < 0.01$; c = $p < 0.001$ compared to + D.

α = $p < 0.05$; β = $p < 0.01$; γ = $p < 0.001$ compared to − D.

remained undetectable (fig. 2). Despite the important increase in plasma iPTH observed in − D mothers at L_{20} (fig. 4), $1,25(OH)_2D$ failed to increase in plasma, reflecting a depletion in maternal vitamin D stores (fig. 2).

Newborn pups from the − D group had decreased plasma concentrations of Ca and P when compared to + D pups, as well as undetectable 25 (OH)D levels (fig. 3). After 20 days of lactation, Ca and P were further decreased in the plasma, and remained lower than levels observed in pups from the + D control group (fig. 3).

Effect of maternal treatment with D_3 or SD_3

— *Mothers.* — When compared to values obtained in − D non-treated mothers, the SD_3-treated group had a slight rise in plasma biochemical parameters between L_1 and L_{12} (fig. 1). At L_{12}, plasma Ca and P levels were intermediate between those

of the − D and + D control groups (fig. 1). In contrast with results obtained in − D non-treated mothers, plasma 25(OH)D_3 and 24,25$(OH)_2D_3$ were detectable in SD_3-treated mothers at L_{12} and 1,25$(OH)_2$D levels were normal (fig. 2). On the other hand, in D_3-treated mothers, a complete normalization of all but one of the plasma biochemical parameters (24,25$(OH)_2D_3$) measured was observed at L_{12} (figs 1 and 2).

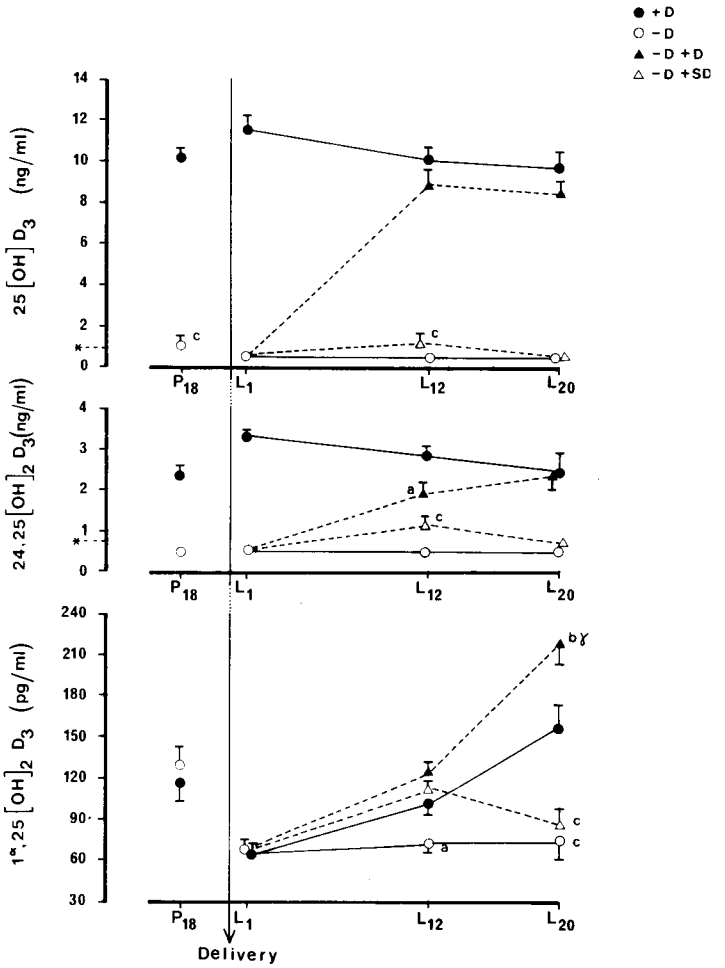

FIG. 2. — *Maternal plasma levels of 25-hydroxyvitamin D_3* (25-OHD), *24,25-dihydroxyvitamin D_3* (24,25$(OH)_2D_3$) *and 1,25-dihydroxyvitamin D_3* (1α, 25$(OH)_2D_3$) *measured in controls* (+ D = D-repleted ; − D = D-depleted) *and treated rats* (− D + D and − D + SD : − D mothers receiving D_3 and SD_3, respectively, during lactation). P_{18} = day 18 of pregnancy ; L_1, L_{12}, L_{20} = days 1, 12 and 20 of lactation respectively.

Results are expressed as mean ± SEM of 5-6 individual determinations. Statistical differences were determined by analysis of variance and Barlett's test.

a : $p < 0.05$; b : $p < 0.01$; c : $p < 0.001$ compared to + D.

γ = $p < 0.001$ compared to − D.

* limit of detection.

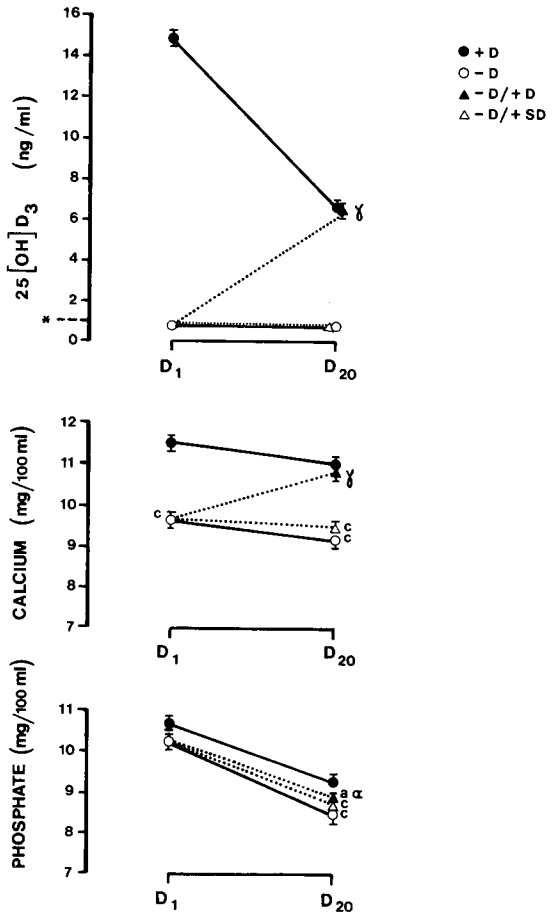

FIG. 3. — *Plasma levels of calcium* (Ca), *phosphorus* (P) *and 25(OH) D in newborn and 20-day old suckling pups from control groups* (+ D = D-repleted mothers, − D = D-depleted mothers) *and treated groups* (− D + D and − D + SD : − D mothers receiving D_3 and SD_3, respectively, during lactation).

Results are expressed as mean ± SEM of 16-20 individual measures/group for Ca and P, 8 pools of 3/4 pups/group for 25-OHD in newborns and 9 individual measures/group for 25-OHD in 20-day old pups. Statistical differences were determined by analysis of variance and Barlett's test.

a : $p < 0.05$; c : $p < 0.001$ compared to + D ; α : $p < 0.05$; γ = $p < 0.001$ compared to − D.

* limit of detection.

In SD_3-treated mothers, the positive changes in the biochemical parameters observed between L_1 and L_{12} were reversed between L_{12} and L_{20}. Plasma levels of Ca, P (fig. 1) and vitamin D metabolites (fig. 2) were clearly decreased at L_{20} when compared to L_{12}, and were not significantly different from those of − D mothers. An increase in plasma iPTH was observed at L_{20} in − D + SD mothers (fig. 4), although it was less than in − D non-treated mothers. By contrast, in the

D_3-treated group most plasma biochemical parameters were normalized at L_{20} (figs 1, 2 and 4). Only 1,25(OH)$_2$D levels remained increased in the plasma of − D + D mothers, when compared to control values of + D mothers.

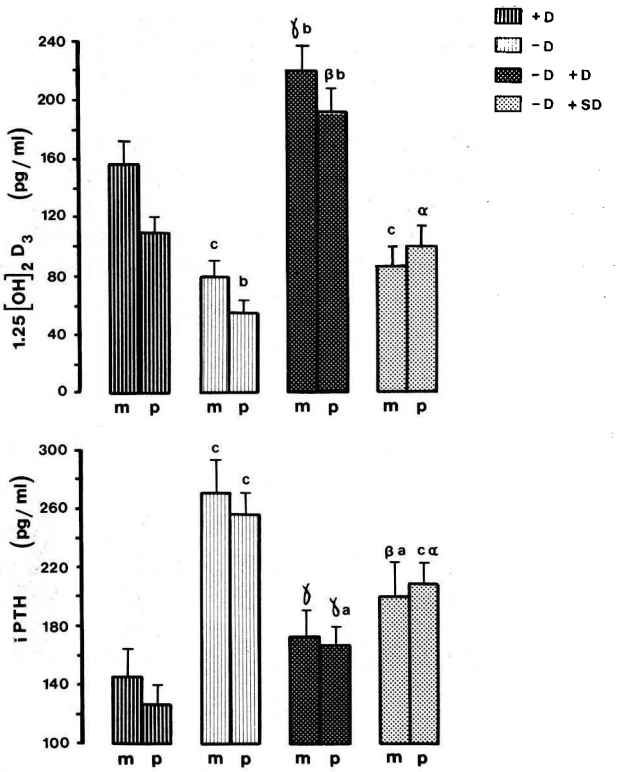

FIG. 4. — *Plasma levels of 1,25 dihydroxyvitamin D_3* (1,25 (OH)$_2$D$_3$) *and parathormone* (PTH) *measured at L_{20} in both mothers* (m) *and suckling pups* (p) *from control groups* (+ D = D repleted mothers, − D = D-depleted mothers) *and treated groups* (− D + D and − D + SD = − D mothers receiving D_3 and SD_3, respectively, during lactation).

Results are expressed as mean ± SEM of 5-6 individual measures/group in mothers or 11-24 individual measures/group in suckling pups. Statistical differences were determined by analysis of variance and Barlett's test.

a : $p < 0.05$; b : $p < 0.01$; c : $p < 0.001$ compared to + D.

α : $p < 0.05$; β : $p < 0.01$; γ : $p < 0.001$ compared to − D.

— *Suckling pups.* — After 20 days of SD_3 maternal treatment, plasma levels of Ca and P were abnormal in pups from this group, in contrast to the results obtained in pups from D_3-treated mothers (fig. 3). Furthermore, plasma levels of 25(OH)D were undetectable in − D + SD pups whereas they were normalized in pups from the − D + D group (fig. 3). Nevertheless, iPTH was higher than normal in pups from both the SD_3 and D_3-treated groups (fig. 4), whereas 1,25(OH)$_2$D levels were clearly increased in pups from the − D + D group but not in those from the − D + SD group when compared to the controls (fig. 4).

B. — *Body weight, bone growth and bone ashes*

— *Mothers.* — In SD_3-treated mothers, body weight (BW), femur length and all bone ash parameters were abnormal at sacrifice (L_{20}) and similar to those obtained in − D non-treated mothers (table 1). By contrast, in D_3-treated mothers, most parameters measured were normalized (table 1).

— *Suckling pups.* — After 20 days of maternal SD_3 supplementation, body weight, femur length and bone mineral content of pups were quite identical to values in − D pups from non-treated mothers (table 1). After maternal D_3 supplementation, only body weight was normalized (table 1). In contrast to results obtained in − D + D mothers, femur length and measured parameters of bone ashes remained abnormal in their pups.

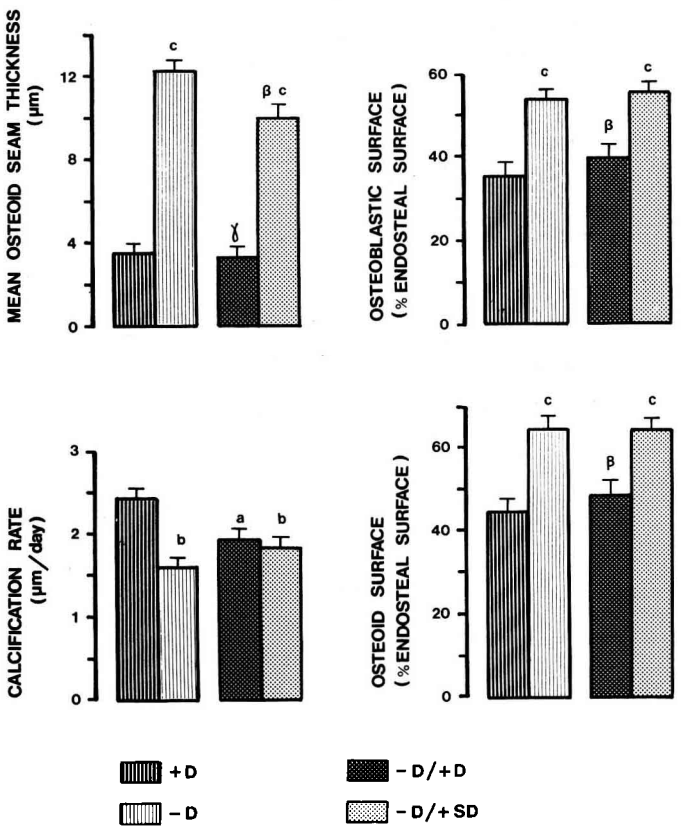

FIG. 5. — *Histomorphometric analysis of maternal caudal vertebrae obtained at day 20 of lactation from control rats* (+ D = D-repleted ; − D = D-depleted) and *treated rats* (− D + D and − D + SD : − D mothers receiving D_3 and SD_3, respectively, during lactation).

Results are expressed as mean ± SEM of 4-6 animals. Statistical differences were determined by analysis of variance and Barlett's test.

a : $p < 0.05$; b : $p < 0.01$; c : $p < 0.001$ compared to + D.

β : $p < 0.01$; γ : $p < 0.001$ compared to − D.

TABLE 1

Body weight, femur length, bone ash and mineral content of bone ash in mothers and pups from control groups (+ D : D-repleted and − D : D-depleted) *and − D-treated groups* (− D mothers vitamin D_3-treated (− D + D) or SD_3-treated (− D + SD) during the lactation period) *at day 20 of lactation.*

	Group	Body weight (g)	Femur length (mm)	Bone ash (% bone dry weight)	Ca (mg/g) bone ash)	P mg/g bone ash)	Mg (mg/g bone ash)
Mothers	+ D (5)	285.0 ± 5.2	35.2 ± 0.2	54.9 ± 0.6	295.4 ± 3.7	154.2 ± 3.3	6.2 ± 0.2
	− D (5)	228.8 ± 5.2**	33.8 ± 0.2*	48.3 ± 0.6**	251.8 ± 3.7**	162.4 ± 3.3°	8.3 ± 0.2**
	− D + D (5)	266.0 ± 5.2°	34.6 ± 0.2	54.3 ± 0.6	287.8 ± 3.7	155.8 ± 3.3	6.2 ± 0.2
	− D + SD (6)	238.0 ± 5.0*	33.5 ± 0.2**	46.9 ± 0.5**	279.0 ± 3.4*	163.5 ± 3.0°	7.8 ± 0.2**
Pups	+ D	49.4 ± 0.6 (23)	13.9 ± 0.2 (14)	43.1 ± 0.5 (22)	350.4 ± 5.2 (17)	208.6 ± 3.7 (17)	7.2 ± 0.3 (17)
	− D	40.8 ± 0.5** (25)	13.0 ± 0.1 (20)	39.5 ± 0.5** (22)	277.3 ± 4.5** (22)	183.7 ± 3.3** (22)	8.4 ± 0.3* (22)
	− D + D	49.1 ± 0.5 (25)	13.4 ± 0.1 (23)	40.1 ± 0.5** (21)	291.0 ± 4.7** (21)	186.9 ± 3.3** (21)	7.9 ± 0.3 (21)
	− D + SD	41.6 ± 0.4** (29)	13.3 ± 0.1** (23)	41.0 ± 0.5* (20)	278.1 ± 4.8** (20)	180.6 ± 3.4** (20)	7.9 ± 0.3 (20)

° $p < 0.05$; * $p < 0.01$; ** $p < 0.001$ compared to + D controls, Barlett's test.
Results are expressed as mean ± SEM.
() number of rats analysed per group.

C. — *Bone histomorphometry*

— *Mothers.* — Despite 20 days of SD_3 treatment during the lactation period, mother rats from this group exhibited histological signs of osteomalacia of a severity similar to that of − D untreated mothers. This was characterized by an increase in the volume, surface and thickness of osteoid tissue associated with a marked decrease in calcification rate when compared to + D control mothers (table 2, fig. 5). These parameters were mainly normalized in − D mother rats after 20 days of D_3 treatment (table 2, fig. 5).

By contrast with data concerning bone mineralization, bone resorption parameters were normalized in mothers from the SD_3-treated group (table 2), although the osteoblastic surface remained abnormal (fig. 5). Both osteoclastic and osteoblastic parameters were normalized in mothers from the D_3-treated group (fig. 5, table 2).

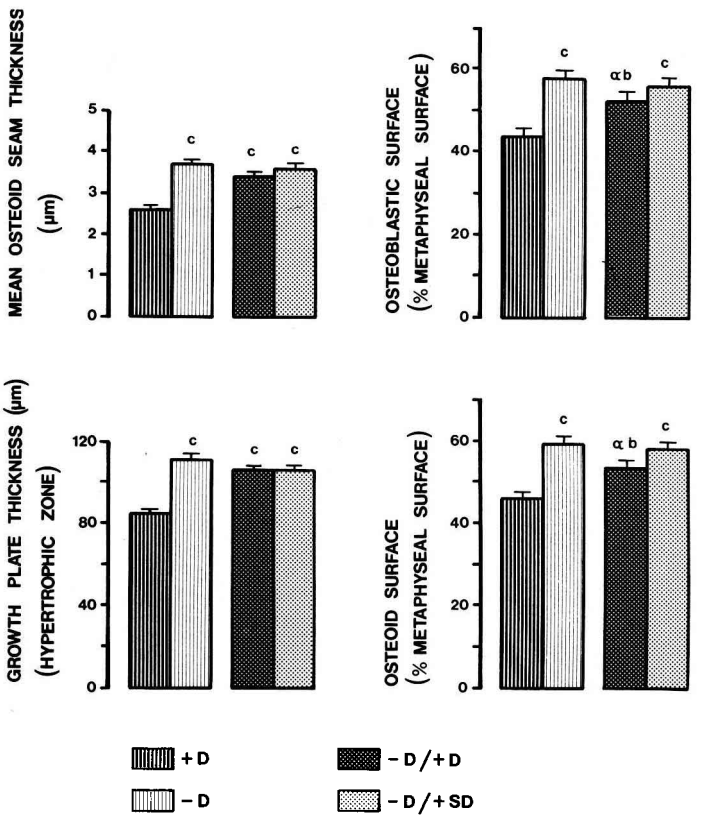

FIG. 6. — *Histomorphometric analysis of caudal vertebrae from 20-day old suckling pups of control groups* (+ D = D-repleted mothers ; − D = D-depleted mothers) *and treated groups* (− D + D and − D + SD : − D mothers receiving D_3 and SD_3, respectively, during lactation).

Results are expressed as mean ± SEM of 8-9 animals. Statistical differences were determined by analysis of variance and Barlett's test.

b : $p < 0.01$; c : $p < 0.001$ compared to + D.

α : $p < 0.05$ compared to − D.

TABLE 2

Histomorphometric measurements of caudal vertebrae in mothers and pups from control groups (+ D : D-repleted and − D : D-depleted) *and − D-treated groups* (− D mothers vitamin D_3-treated (− D + D) or SD_3-treated (− S + SD) during the lactation period) *at day 20 of lactation.*

Group		CBV (% BT)	VO (% BT)	DL (% ST)	MLT (% days)	Ost. S (% ST)	N° Oc/mm2 bone
Mothers	+ D (5)	10.5 ± 0.9	1.1 ± 0.2	22.6 ± 1.8	1.5 ± 0.5	7.3 ± 0.8	6.7 ± 0.7
	− D (5)	5.2 ± 0.9**	2.6 ± 0.2**	6.7 ± 1.8**	8.6 ± 0.5**	12.2 ± 0.8**	10.8 ± 0.7**
	− D + D (5)	11.9 ± 0.9	1.2 ± 0.2	17.8 ± 1.8*	1.8 ± 0.5	7.7 ± 0.8	8.0 ± 0.7
	− D + SD (6)	5.1 ± 0.9**	1.9 ± 0.2*	10.2 ± 1.6**	5.8 ± 0.5**	8.2 ± 0.8	7.1 ± 0.6
Pups	+ D (8)	15.1 ± 0.7	1.7 ± 0.15	—	—	6.5 ± 0.6	20.4 ± 1.6
	− D (9)	15.3 ± 0.7	3.5 ± 0.14**	—	—	10.0 ± 0.6**	29.0 ± 1.5**
	− D + D (9)	15.6 ± 0.7	1.7 ± 0.14	—	—	10.3 ± 0.6**	26.9 ± 1.5*
	− D + SD (9)	14.8 ± 0.7	2.6 ± 0.14**	—	—	9.6 ± 0.6**	25.6 ± 1.5°

CBV : calcified bone volume ; BT : bone tissue ; VO : volume of osteoïd tissue ; DL : double-labelled surface ; MLT : mineralization lag-time ; ST : total bone surface ; Ost S : Osteoclastic surface ; N° OC : osteoclast number.

° $p < 0.05$; * $p < 0.01$; ** $p < 0.001$ compared to + D controls, Barlett's test.

Results are expressed as mean ± SEM.

() : number of rats analysed per group.

— *Suckling pups.* — Bone lesions remained evident in pups from both the SD_3 and D_3-treated groups, in contrast to results obtained in their mothers. Decreased mineralization of bone and cartilage was indicated by an increase in osteoid tissue associated with increased thickness of the hypertrophic zone of the growth plate (table 2, fig. 6). In addition pups from both treated groups compared to values in the + D control pups presented increased osteoclastic bone surface and osteoclast number (table 2) associated with increased osteoblastic surface (fig. 6).

Discussion.

The results of this study show that vitamin D_3-3β sulfate (1 300 pmoles) is clearly less active than the same dose of free vitamin D_3 in promoting normal mineral homeostasis and bone mineralization in rats during the lactation period.

Lactation is a physiological period when maternal calcium stress is augmented due to high calcium demands for milk synthesis (32-34). In rats, this is particularly evident since the calcium transfer through milk is one hundred-fold greater than urinary calcium excretion (35). Because of these high demands, the lactating rat must provide minerals from bone stores in order to maintain normal Ca and P transfer (32,36). Some adaptive mechanisms are evident during the reproductive stages, including augmented food intake and increased intestinal mineral absorption, but they are not sufficient to provide adequate amounts of minerals for milk synthesis. Consequently, vitamin D-sufficient lactating rats lose between 10 % and 20 % of their bone calcium stores (37,38). Thus, lactating rats are very sensitive to any changes in vitamin D balance (38). In the present study we have taken advantage of this physiological adaptation to evaluate the biological activity of SD_3 during the lactation period.

Despite the small amounts of D_3 administered before pregnancy and the reduction of the number of pups per litter to five, − D lactating rats suffered a dramatic decrease in plasma levels of calcium during the lactating period, by contrast to results obtained both during pregnancy (P_{18}) and after delivery (L_1). These data again illustrate the importance of calcium needs during lactation in rats (35,39). In contrast with pregnancy (P_{18}), $1,25(OH)_2D_3$ levels failed to increase during the lactation period in − D mothers, most likely reflecting depressed vitamin D stores, as shown by the non-detectable levels of plasma 25(OH)D and $24,25(OH)_2D$ during lactation. However, $1,25(OH)_2D$ remained detectable in plasma of − D mothers, probably due to the persistence of trace amounts of $25(OH)D_3$. Nevertheless, the existence of a severe osteomalacia associated with secondary hyperparathyroidism at L_{20} of lactation in − D mothers was clearly indicative of D deficiency.

In − D mother rats, there was a noticeable improvement in plasma levels of Ca, P and vitamin D metabolites after SD_3 treatment. This suggests that treatment retarded the evolution of the D-deficiency state during the first 12 days of lactation. It is probable that there was a partial « *in vivo* » hydrolysis of SD_3 at the beginning of the lactation period in the mother rats, as previously reported by Le Boulch *et al.* (20) in lactating rats after a single intravenous injection of

radiolabelled SD_3. The partial hydrolysis of SD_3 into D_3 is nevertheless of minor importance since, except for $1,25(OH)_2D$, the parameters were not normalized, and 25(OH)D was only slightly increased in the plasma of − D + SD mothers after 12 days of treatment. These results are in contrast with those obtained with an equimolar treatment of free vitamin D_3.

This positive effect of SD_3 treatment was abolished during the last week of lactation, and at L_{20} − D + SD mothers showed plasma and bone abnormalities similar to the − D non-treated control mothers. A decreased hydrolysis of SD_3, an increase in vitamin D demands or the co-existence of both phenomena could be responsible for this effect.

Despite vitamin D deficiency, SD_3-treated mother rats showed histomorphometric evidence of normal bone resorption. This finding corroborates our previous observations made in SD_3-treated post-pregnant rats (22). It is possible that discontinuous administration of SD_3 produced a delay in SD_3 activity, leading to an irregular profile of associated calciotropic hormones such as PTH. Parallel changes would occur in bone metabolism and the bone resorption picture that we obtained at the time of sacrifice may, in fact, reflect the postulated fluctuation of PTH production. Nevertheless, − D + SD mother rats were much closer, biochemically and histologically, to D-depleted untreated mothers, than to the − D mother rats given free vitamin D_3 treatment, in which most of the parameters studied were normalized. Thus, the effectiveness of free D_3 contrasted markedly with that of equimolar SD_3.

In accordance with maternal results, pups from the SD_3-treated group showed plasma biochemical parameters comparable to − D control pups. By contrast, all plasma biochemical parameters in pups from the − D + D group were either normalized or clearly improved. Nevertheless, in pups submitted to a D-deficient fetal life, neither D_3 nor SD_3 maternal treatment during the suckling period could reverse their bone abnormalities, which remained mainly comparable to those observed in the − D control pups.

It was previously shown that reduced food consumption occurring in − D rats led to impaired milk secretion which, in turn, was responsible for mineral abnormalities of suckling pups (40). A maternal vitamin D_3 treatment which normalized mineral metabolism in mothers also led to normal maternal food consumption and normal milk secretion, thus inducing normalization of growth and mineral metabolism as evaluated by serum calcium, femur length and body weight (41). Accordingly, we should have observed a complete normalization of both plasma and bone parameters, at least in pups from the − D + D group since maternal mineral metabolism was normalized in this group. However, bone abnormalities were not corrected either in this group (− D + D) or in pups from SD_3-treated mothers. These observations demonstrate the low activity of SD_3 on mineral metabolism in pups as well as in mothers, and therefore the hypothesis of an important suckling-related activity of SD_3 can be discarded. In addition, normalization of maternal mineral metabolism is not sufficient to correct bone metabolism in suckling pups. Larger doses of vitamin D_3 may perhaps be necessary to normalize mineral and bone lesions in both mothers and suckling pups. Our results support the suggestion that vitamin D stores repleted before

birth may be important for bone development during suckling, since similar abnormalities were detected in all three groups where pups suffered from an equally D-deficient fetal life. This hypothesis is in agreement with results obtained recently by Clemens and Fraser (42) who showed that rat pups store vitamin D as 25(OH)D and $24,25(OH)_2D$ during fetal life and that these stores are their major source of vitamin D during suckling. In fact, vitamin D does not appear to play a major role in the skeletal development of the rat foetus (43), probably because placental calcium and phosphorus transport are not impaired in the absence of vitamin D (44) and do not depend on their maternal plasma levels (45,46). If fetal life can take place in a D-deficient state in nearly normal conditions, the vitamin D stored during fetal life could be of major importance for normal post-natal bone development.

These results show that vitamin D_3-3β sulfate has a low biological activity during the lactation period, although a small « *in vivo* » hydrolysis of SD_3 was detected in mothers. These data are in agreement with previous reports concerning male young rats (17,18). In contrast with previous suggestions (6-8,21) however, no important activity was detected for SD_3 during the reproductive stages. Accordingly we and others (10, 13, 47) have recently shown that SD_3, although present in human milk, is found in clearly lower concentrations than previously reported and that the SD_3 present in maternal milk, does not seem to affect the vitamin D stores of exclusively breast-fed babies, by contrast with the 25(OH)D content of maternal milk (47).

Altogether these results provide strong evidence that sulfoconjugated vitamin D_3 has no biological activity on bone and mineral metabolism, even during the reproductive period.

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Résumé. *Le 3β-sulfate de vitamine D_3 n'a pas d'effet biologique chez la ratte allaitante carencée en vitamine D.*

Nous avons montré précédemment que l'administration de 3 β-sulfate de vitamine D_3 (SD_3) affecte peu le métabolisme phosphocalcique chez la ratte gestante. Dans ce travail nous avons comparé l'activité biologique de la vitamine D_3 libre (D_3) et du S_3 pendant l'allaitement. A la mise-bas, des rattes carencées en vitamine D (− D) ont été traitées tous les deux jours par 1 300 pmoles de D_3 ou de SD_3 pendant l'allaitement. Les taux plasmatiques de vitamine D ont été mesurés avant, pendant et à la fin du traitement chez les mères et à un jour et 20 jours de vie chez les ratons allaités. Les mères et les ratons ont été sacrifiés au 20e jour d'allaitement et on a procédé à une évaluation hormonale biochimique et à une analyse minérale et histomorphométrique des os. Après 12 jours de traitement par le SD_3 chez les mères carencées on a observé une augmentation légère mais significative de la calcémie, de la phosphatémie et des métabolites de la vitamine D.

Cet effet s'est annulé à la fin de l'allaitement, où la plupart des paramètres plasmatiques étaient identiques à ceux des mères − D non traitées. Par contre, l'administration de

D_3 pendant 20 jours chez les mères – D a complètement normalisé les paramètres biochimiques. Ces résultats ont été confirmés par l'analyse des paramètres statiques et dynamiques de la formation osseuse. Chez les ratons allaités de mères traitées par la SD_3, aucune amélioration des paramètres histologiques et biochimiques de formation et de résorption osseuse n'a été observée, ces paramètres restant comparables à ceux des ratons – D. Par contre, chez les ratons de mères traitées par la D_3 la plupart des paramètres biochimiques ont été normalisés alors que le métabolisme osseux n'a pas été entièrement normalisé. Ces résultats démontrent que l'activité biologique du SD_3 sur le métabolisme osseux et minérale est très faible chez le rat pendant la période d'allaitement.

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