Influence of calcium or 1,25-dihydroxyvitamin D3 supplementation on the hepatic microsomal and in vivo metabolism of vitamin D3 in vitamin D-depleted rats.

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Research Article

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Influence of Calcium or 1,25-Dihydroxyvitamin D₃ Supplementation on the Hepatic Microsomal and In Vivo Metabolism of Vitamin D₃ in Vitamin D–depleted Rats

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Abstract

Hypocalcemic vitamin D (D)–depleted rats were supplemented with calcium or 1,25(OH)₂D₃, and the metabolism of D₃ to 25(OH)D₃ was studied. Infusion with 7 or 65 pmol 1,25(OH)₂D₃ · 24 h⁻¹ led to normal or slight hypercalcemia associated with physiological and supraphysiological plasma concentrations of the hormone while calcium supplementation normalized plasma calcium despite 1,25(OH)₂D₃ concentrations as low as those observed in hypocalcemic controls. Constant administrations of [¹⁴C]D₃ during the supplementation regimens uncovered a stimulation of the in vivo 25(OH)D₃ production by calcium supplementation; this was further confirmed in vitro by an increase in the hepatic microsomal D₃-25 hydroxylase. The group supplemented with pharmacological doses of the hormone displayed lower circulating concentrations of both D₃ and 25(OH)D₃ while the in vitro 25(OH)D₃ production remained unaffected by 1,25(OH)₂D₃. Investigation of the kinetics of intravenous 25(OH)[³H]D₃ revealed similar elimination constants in all groups. The data indicate that calcium supplementation of hypocalcemic D–depleted rats results in an increased transformation of D₃ into 25(OH)D₃ while supplementation with 1,25(OH)₂D₃ does not affect the in vitro D₃-25 hydroxylase but seems to influence the in vivo handling of the vitamin by accelerating its metabolism.

Introduction

Vitamin D₃ (D₃)[1] is a secosteroid produced in the skin from 7-dehydrocholesterol (1). Once in circulation, D₃ is taken up by the liver and hydroxylated at C-25 by a cytochrome P-450–dependent mixed function oxidase system. Two independent enzyme systems have been described in the microsomal (2) and mitochondrial (3) fractions, respectively. In view of its lower K_m

value (4), the microsomal D₃-25 hydroxylase is considered as being physiologically more significant in the rat than the mitochondrial enzyme. The hepatic metabolite, however, 25-hydroxyvitamin D₃ [25(OH)D₃], has only weak biological activity and requires further hydroxylation at the C-1α position before achieving its full hormonal potential. This reaction is catalyzed by a renal mitochondrial cytochrome P-450–dependent mixed function oxidase system, which is tightly regulated by stimulatory factors (hypocalcemia, parathyroid and growth hormones) associated with states of calcium demand as well as by the inhibitory regulation of hypercalcemia and 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) itself (5, 6). However, unlike that of the renal 25(OH)D₃-1α-hydroxylase, the regulation of the hepatic D₃-25 hydroxylase is still rather ill defined. Early work by Bhattacharyya and De Luca (7) demonstrated that previous dosing with D₃ diminished the liver's subsequent capacity to hydroxylate tritiated substrate. Later experiments by Rojanasathit and Haddad (8), however, showed that the apparent in vivo hepatic limitations in 25(OH)D₃ production could be overcome by increased substrate concentrations. Other groups (9–11) were also unable to confirm the importance of the vitamin D status in the control of the D₃-25 hydroxylase activity in the liver. On the other hand, two recent reports have reopened the question of the regulation of the D₃-25 hydroxylase. Indeed, following in vitro experiments in rat liver preparations (12) as well as following oral administration in humans (13), the possibility has been raised that 1,25(OH)₂D₃ may be an inhibitor of the hepatic D₃-25 hydroxylase activity.

The aim of the present studies was therefore to examine the differential effects of the hormone 1,25(OH)₂D₃ and of its associated physiological influence on the extracellular calcium concentration on the in vivo and in vitro metabolism of D₃ to 25(OH)D₃ in vitamin D–depleted rats.

Methods

Animals and treatment procedures

Nurturing female Sprague-Dawley rats with 14-d-old litters (Charles River, Ltd., St. Constant, Quebec, Canada) were housed in plastic wire-topped cages and fed ad lib. a vitamin D–deficient diet (14) containing 0.2 and 0.18%, respectively, of elementary calcium and phosphorus. Upon weaning, male rats were housed in hanging stainless steel wire cages and fed ad lib. the vitamin D–deficient diet as well as demineralized water for a period of 5–7 wk before being randomly assigned to oral calcium or 1,25(OH)₂D₃ supplementation regimens. Oral calcium supplementation was achieved by supplying a 3% solution of calcium gluconate as drinking water. Hormonal supplementation was achieved through the intraperitoneal implantation of osmotic minipumps (Alza Corp., Palo Alto, CA) which delivered constant infusions of 1,25(OH)₂D₃ at doses of 7 or 65 pmol · 24 h⁻¹. Control animals (as well as calcium supplemented ones) were implanted with minipumps containing only the vehicle (ethanol/propylene glycol/saline, 15:65:20).

1. *Abbreviations used in this paper:* ANOVA, analysis of variance; D₃, vitamin D₃; HPLC, high performance liquid chromatography; 25(OH)D₃, hydroxyvitamin D₃; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃.

Serum parameters of vitamin D depletion and of supplementation regimens

Serum vitamin D metabolites were measured after extraction and chromatography as described earlier (15). 25(OH)D was analyzed by a competitive protein binding assay using sheep serum in a dilution of 1:40,000 as binding protein; the lower limit of sensitivity of the method was 10 pg · ml^{-1}. 1,25(OH)$_2$D$_3$ was measured by a competitive protein binding assay using a cytosolic receptor from bovine thymus (16); the lower limit of sensitivity of the method was 1 pg · ml^{-1}. Plasma calcium was measured by the colorimetric method of Gindler and King (17). In order to evaluate the effect of the high 1,25(OH)$_2$D$_3$ dose on the renal function, the plasma creatinine and blood, urea nitrogen (BUN) concentrations were determined in controls and in the 65-pmol · 24 h^{-1} group by standard methods adapted for multianalyzer analysis.

In vivo metabolism of [^{14}C]D$_3$

After 7 d of hormonal supplementation or 10 d of calcium supplementation, animals from all groups were implanted with a second osmotic minipump delivering a constant physiological dose of 3.52 nmol [^{14}C]vitamin D$_3$ · 24 h^{-1} ([4-^{14}C]vitamin D$_3$, 57 mCi · mmol^{-1}, Amersham Corp., Oakville, Ontario, Canada). An initial loading dose of 5.2 nmol [^{14}C]D$_3$ was administered to all rats as a single intravenous injection immediately after minipump implantation. Blood was drawn into Natelson collecting tubes (Fisher Scientific, Ville Mont-Royal, Quebec, Canada) from tail cuts 8 h and every 24 h following initiation of [^{14}C]D$_3$ infusion. On the day the animals were killed (corresponding to day 13 of the ongoing supplementation regimens and to 144 h of [^{14}C]D$_3$ infusion) all rats were injected with a single intravenous dose of 15 pmol of 25-hydroxy-[^{3}H]vitamin D$_3$ (25-hydroxy[23,24(n)-^{3}H]vitamin D$_3$, 100 Ci · mmol^{-1}, Amersham Corp.) to evaluate its elimination kinetics. 100–200 μl of blood were drawn into heparinized syringes from the jugular vein at 2, 5, and 10 min following 25-hydroxy-[^{3}H]vitamin D$_3$ (25(OH)-[^{3}H]D$_3$) administration while later blood samples (30, 60, 120, and 240 min) were collected from tail cuts. 10 min before the time the animals were killed, ^{131}I-labeled human serum albumin (200,000 cpm in 200 μl of a 5% solution of human serum albumin) was injected intrajugularly in order to evaluate the plasma volume and the plasma contamination of the tissues and organs studied. All animals were sacrificed 420 min after 25(OH)-[^{3}H]D$_3$ injection by exsanguination through the abdominal aorta, under light ether anesthesia. Duplicate plasma aliquots of all samples were counted in 6 ml Biofluor (New England Nuclear, Boston, MA) in a liquid scintillation spectrometer (model LS 3801, Beckman Instruments, Inc., Palo Alto, CA).

Upon death of the animal, the liver was flushed with isotonic saline, excised, and weighed. The following tissues were also excised bilaterally and weighed: kidneys, epidydimal fat pads, quadratus lumborum muscle (of the deep pelvic musculature), as well as the overlaying abdominal white adipose tissue. Whole or aliquots of the various tissues and organs as well as triplicate aliquots of the final plasma samples were counted in a spectrometer (model Gamma 8000, Beckman Instruments, Inc.) to evaluate ^{131}I activity. All tissues were then frozen at −20°C.

Tissue homogenates were later prepared using a Kinematica Polytron tissue grinder (Brinkmann Instruments, Rexdale, Ontario, Canada) with isotonic saline in the following proportions: kidneys, 33%; muscle, 25%; epidydimal fat, 40%; and abdominal fat, 40%, wt/vol, respectively. Liver homogenates were prepared with 4 vol of 0.25 M sucrose using a Potter-Elvehjem type glass-Teflon homogenizer. Aliquots of tissue homogenates were counted in 10 ml Biofluor following refrigeration to diminish chemiluminescence.

Separation of [^{14}C]D$_3$ and 25(OH)-[^{14}C]D$_3$ from plasma. Plasma samples were extracted with 15 ml ethyl acetate:toluene (9:1, vol/vol). Radioinert D$_3$ (Sigma Chemical Co., St. Louis, MO) (1 μg) and 25(OH)D$_3$ (1 μg) were added as internal standards and carriers and the final extraction mixture was vortexed with 0.15 g ammonium carbonate and 1 ml distilled water. Following centrifugation, the organic phase was collected while the aqueous phase was extracted two more times using 10 ml ethyl acetate/methanol (9:1, vol/vol). The combined organic phases were evaporated

under a stream of nitrogen. The plasma extracts were then applied to Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden) columns (0.9 × 18 cm) and eluted with chloroform/n-hexane (62:38, vol/vol) to isolate [^{14}C]D$_3$ and 25(OH)-[^{14}C]D$_3$ from lipid contaminants. More polar metabolites such as the dihydroxylated derivatives were not collected because, at the dose of [^{14}C]D$_3$ administered, the [^{14}C]D$_3$ specific activity did not allow reliable detection.

The portion containing [^{14}C]D$_3$ and 25(OH)-[^{14}C]D$_3$ was collected, dried under nitrogen, dissolved in n-hexane/isopropanol (96:4, vol/vol) and injected into a high performance liquid chromatograph (HPLC) (model 100 A, Beckman Instruments, Inc.) fitted with a Zorbax-Sil column (25 cm × 4.5 mm i.d.) (Dupont Instruments, Wilmington, DE); the samples were eluted at a flow rate of 2.5 ml · min^{-1} with n-hexane/isopropanol (96:4, vol/vol) to obtain [^{14}C]D$_3$ at 5.4 min and 25(OH)-[^{14}C]D$_3$ at 12.8 min with no overlap between the two compounds. After 16 min, the polarity of the solvent system was gradually increased to n-hexane/isopropanol 80:20 (vol/vol) over 2 min and kept at that level for 10 min. This procedure served the purpose of harvesting the radioactivity associated with other polar vitamin D$_3$ derivatives not eluting under the 25(OH)-[^{14}C]D$_3$ peak. The nature of the metabolite(s) eluting in this portion of the chromatogram (thereafter called "polar peak") was not investigated. Fractions of 1 min were collected and counted in 6 ml Biofluor. The identity of the putative 25(OH)-[^{14}C]D$_3$ produced was established by comigration with crystalline 25(OH)D$_3$ standard in the above HPLC system as well as by reverse-phase HPLC using a C-18 Vydac (Mandel Scientific, Ville St. Pierre, Quebec, Canada) column (25 cm × 4.6 mm i.d.) eluted with acetonitrile:water (80:20, vol/vol) at a flow rate of 1.5 ml · min^{-1}. Overall ^{14}C recovery was calculated to be 69.1±7.3%, and variations in recovery were not related to sampling time or supplementation regimens.

Separation of [^{14}C]D$_3$, 25(OH)-[^{14}C]D$_3$ and 25(OH)-[^{3}H]D$_3$ from liver homogenates. Samples of liver homogenate (5 ml) were extracted using the procedure described above for the plasma samples with the following modifications: 20 ml of the extraction solvent system was used while no internal standards, ammonium carbonate, or distilled water were added. The combined organic phases were evaporated under a stream of nitrogen. The liver extracts were dissolved in hexane and applied to silicic acid (Bio-Sil HA, Bio-Rad Laboratories, Richmond, CA) columns (0.6 × 5 cm in pasteur pipettes) and eluted with an isocratic gradient of hexane/ether (20, 40, 60, and 100% ether, vol/vol) followed by a final methanol wash. Fractions of 1 ml were collected and counted in 5 ml of Ready-Solv HP/b (Beckman Instruments, Inc., Galway, Ireland) using two channels appropriate for ^{14}C and ^{3}H detection. All calculations were made after data reduction from counts per minute to disintegrations per minute and correction for energy spillover of each isotope. Column calibration was obtained with radioactive D$_3$ and 25(OH)D$_3$ standards added to, and extracted from the liver homogenate of an untreated vitamin D–depleted rat. The identity of the compounds recovered under the D$_3$ and 25(OH)D$_3$ peaks was further confirmed by comigration with pure crystalline standards in the straight-phase HPLC system described above. Radioactivity associated with peaks more polar than 25(OH)D$_3$ in the silic acid chromatograms was quantitated but not identified. Overall isotope recovery was 70.1±6.2% for ^{14}C and 87.5±3.7% for ^{3}H with variations in recovery being independent of supplementation regimens.

In vitro metabolism of [^{3}H]D$_3$

In a second series of experiments, animals were pretreated as described above but were killed by exsanguination through the abdominal aorta under light ether anesthesia after 7 d of 1,25(OH)$_2$D$_3$ supplementation or 10–14 d of calcium supplementation. The livers were immediately flushed with ice-cold saline, excised, and a homogenate was then prepared with 4 vol of 0.25 M sucrose at 4°C using a Potter-Elvehjem type glass-Teflon homogenizer. The homogenate was immediately centrifuged on a J-21 centrifuge (Beckman Instruments, Inc.) and the cell debris and nuclei sedimented at 600 g for 10 min and the mitochondria at 15,700 g for 15 min. The supernatant was then centrifuged on an ultracentrifuge (L5-75, Beckman Instruments, Inc.) and the microsomal fraction obtained as the 105,000-g pellet. The microsomal pellet was washed once by re-

suspension in ice-cold 0.15 M KCl followed by recentrifugation. The microsomal pellet was then covered with 2 cm of 0.25 M sucrose and immediately frozen at $-78°C$.

Determination of the hepatic microsomal vitamin D_3-25 hydroxylase activity. Cytochrome P-450 content was determined on the 105,000-g pellet resuspended in 0.1 M potassium phosphate buffer, pH 7.4, using an isosbestic point of 490 nm ($\xi = 91$ nM $\cdot$ cm^{-1}) as described by Omura and Sato (18). All cytochrome P-450 determinations were made on an Aminco DW-2 dual beam spectrophotometer (American Instruments Co., Silver Spring, MD). All enzyme assays were performed on microsomal suspensions as previously described (19). Essentially, suspensions containing 15 mg microsomal protein were preincubated with 26 pmol [^{3}H]vitamin D_3 ([1α,2α(n)-^{3}H]vitamin D_3, 18.3 Ci $\cdot$ mmol^{-1}) for a period of 1 h at 22°C. The reaction mixture was then brought to 37°C in the presence of 5 μmol mercaptoethylamine hydrochloride (thiol group protector) for a period of 5 min and the incubation started by the addition of a buffered NADPH-generating system. Blank incubations were carried out under identical conditions with boiled microsomes or without addition of the NADPH-generating system. The reaction was stopped after 40 min by the addition of 15 ml ethyl acetate/toluene (9:1, vol/vol). Extractions were then carried out as described above for plasma samples with the exception that 15 ml ethyl acetate/methanol (9:1, vol/vol) were used for the two last steps.

Separation of [^{3}H]D_3 and 25(OH)-[^{3}H]D_3 from microsomes. The microsomal extracts were applied to Sephadex LH-20 columns and eluted with chloroform/n-hexane (62:38, vol/vol) to remove lipid contaminants. Portions containing [^{3}H]D_3 and 25(OH)-[^{3}H]D_3 were collected, dried under nitrogen, dissolved in n-hexane/isopropanol (97.5:2.5, vol/vol), and submitted to HPLC as described above with the following modifications: separation of [^{3}H]D_3 and 25(OH)-[^{3}H]D_3 was achieved at a flow rate of 2.5 ml $\cdot$ min^{-1} with n-hexane/isopropanol (97.5:2.5, vol/vol). In this system, [^{3}H]D_3 eluted at 6.5 min and 25(OH)-[^{3}H]D_3 at 19.6 min. Fractions of 1 min were collected and counted in 6 ml Biofluor in a liquid scintillation spectrometer.

Statistical analysis

Data are presented as means±SEM unless otherwise specified. The effects of supplementation regimens were analyzed either by a 2 × 4 factorial analysis of variance (initial/final vs. treatments) or by simple analysis of variance according to Winer (20). Orthogonal contrasts between subgroups were calculated by the method of Dunnett (21). Comparisons of the slopes of 25(OH)-[^{3}H]D_3 elimination curves were done as described by Kleinbaum and Kupper (22).

Results

Systemic effects of calcium and 1,25(OH)$_2$D$_3$ supplementation. Fig. 1 A presents the circulating concentrations of 1,25(OH)$_2$D$_3$ obtained in vitamin D–depleted rats after 7 d of constant intraperitoneal infusion of vehicle or of doses of 7 or 65 pmol 1,25(OH)$_2$D$_3$ $\cdot$ 24 h^{-1}. 1,25(OH)$_2$D$_3$ infusion for a week led to dose-related increases in the plasma concentrations of the hormone to levels of 142.8±34.5 and 354±58.8 pg $\cdot$ ml^{-1} for the 7 and 65 pmol 1,25(OH)$_2$D$_3$ $\cdot$ 24 h^{-1} groups, respectively, as compared with the vehicle-infused control value of 21.6±2.8 pg $\cdot$ ml^{-1}. Calcium supplemented rats exhibited plasma 1,25(OH)$_2$D$_3$ concentrations of 17.8±3.8 pg $\cdot$ ml^{-1}, which were not significantly different from controls. The plasma calcium concentrations are presented in Fig. 1 B. From initially equivalent hypocalcemic states in all groups, 1,25(OH)$_2$D$_3$ infusion at doses of 7 and 65 pmol $\cdot$ 24 h^{-1} for 7 d led to normocalcemic and slightly hypercalcemic states, respectively, while vehicle-infused controls remained hypocalcemic. Oral calcium supplementation, on the other hand, was successful in normalizing plasma calcium despite low plasma 1,25(OH)$_2$D$_3$ concentrations.
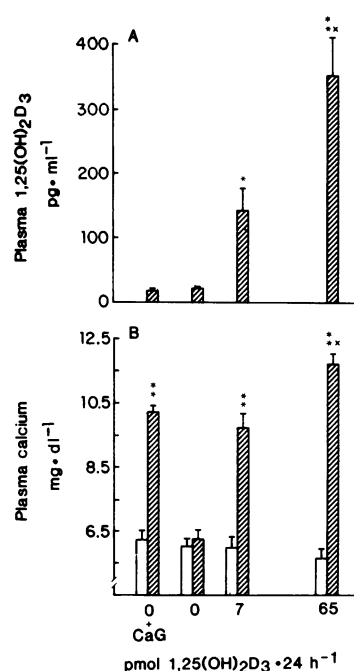

Figure 1. Effect of oral calcium or hormonal supplementation on plasma concentrations of 1,25(OH)$_2$D$_3$ (A) and total plasma calcium (B) in vitamin D–depleted rats. Calcium-supplemented rats (O + CaG) received oral calcium gluconate and intraperitoneal vehicle infusion for 10–14 d. Controls (o) received intraperitoneal vehicle infusion for 1 wk while hormonally supplemented animals received intraperitoneal infusions of 7 or 65 pmol 1,25(OH)$_2$D$_3$ $\cdot$ 24 h^{-1} during the same period. Results are expressed as means±SEM, $n = 28$ or seven rats per subgroup (A) and $n = 40$ or 10 rats per subgroup (B). Normal rat serum 1,25(OH)$_2$D$_3$ concentrations range from 48 to 75 pg $\cdot$ ml^{-1} in the authors' laboratory. Statistically significant differences between group means were analyzed by ANOVA (A) and by a 2 × 4 factorial analysis of variance (B). Statistically significant differences of individual comparisons between subgroups were: different from controls (o), *$P < 0.05$, **$P < 0.005$; different from 7 pmol 1,25(OH)$_2$D$_3$ $\cdot$ 24 h^{-1}, $^x P < 0.001$. In (B) all final calcium concentrations (striped bars) were statistically greater than initial ones (open bars) ($P < 0.001$) except in the control group.

Plasma 25(OH)D$_3$ concentrations were not influenced by the supplementation regimens and were found to be undetectable in one-third of the samples while remaining below 0.4 ng $\cdot$ ml^{-1} in all other samples.

Upon initiation of the supplementation regimens, body weights were similar in all treatment groups (230±6 g for unsupplemented controls and 237±5, 239±11, and 241±10 g for calcium-supplemented, 7 and 65 pmol 1,25(OH)$_2$D$_3$ $\cdot$ 24 h^{-1} groups, respectively, NS). However, they increased significantly more following 7 d of exogenous 1,25(OH)$_2$D$_3$ infusion than following that of vehicle, or 10 d of oral calcium supplementation (275±7 and 271±9 g for the 7 and 65 pmol 1,25(OH)$_2$D$_3$ $\cdot$ 24 h^{-1} groups vs. 250±10 and 251±11 g for the unsupplemented controls and calcium-supplemented animals, respectively, $P < 0.05$). Simi-larly, liver weights were found to be significantly larger ($P < 0.05$) in the 7 and 65 pmol 1,25(OH)$_2$D$_3$ $\cdot$ 24 h^{-1} groups (3.78±0.11 and 3.84±0.08 g $\cdot$ 100 g body wt^{-1}, respectively) than in vehicle-infused or calcium-supplemented rats (3.58±0.12 and 3.51±0.12 g $\cdot$ 100 g body wt^{-1}, respectively). The 1,25(OH)$_2$D$_3$-mediated increases in body as well as liver weights were, however, not influenced by the dose of hormone infused.

The high 1,25(OH)$_2$D$_3$ dose did not seem to have any deleterious effect on the renal function as evidenced on the day the animals were killed, by plasma creatinine values of 0.43±0.04 and 0.39±0.07 mg $\cdot$ dl^{-1} and BUN values of 12.8±1.3 and 14.6±1.2 mg $\cdot$ dl^{-1} for controls and the 65 pmol 1,25(OH)$_2$D$_3$ $\cdot$ 24 h^{-1} group, respectively.

In vivo metabolism of [^{14}C]vitamin D_3. Fig. 2 illustrates the effect of [^{14}C]D$_3$ infusion on the plasma calcium concentrations of controls, and rats pretreated with oral calcium or intraperi-
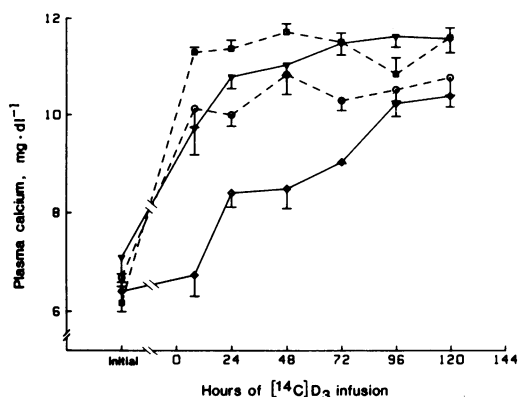
Figure 2. Effect of [^{14}C]D$_3$ infusion on plasma concentrations in calcium- and hormone-infused vitamin D–depleted rats. Initial values refer to the time prior to initiation of oral calcium gluconate (▼——▼), control vehicle-infusion (◇——◇) and infusion with 7 (o - - - o) or 65 pmol 1,25(OH)$_2$D$_3$·24 h^{-1} (□ - - - □). Time 0 corresponds to 10 d of calcium supplementation or 1 wk of hormone or vehicle infusion. Note that all groups achieve stable plasma calcium concentrations from 96 h of [^{14}C]D$_3$ infusion on.

toneal 1,25(OH)$_2$D$_3$ infusion. Before the supplementation regimens, rats in all groups had an equivalent hypocalcemic and vitamin D–depleted status as indicated by the initial plasma calcium and 25(OH)D$_3$ concentrations. Unsupplemented controls were still hypocalcemic 8 h after the onset of [^{14}C]D$_3$ ad-
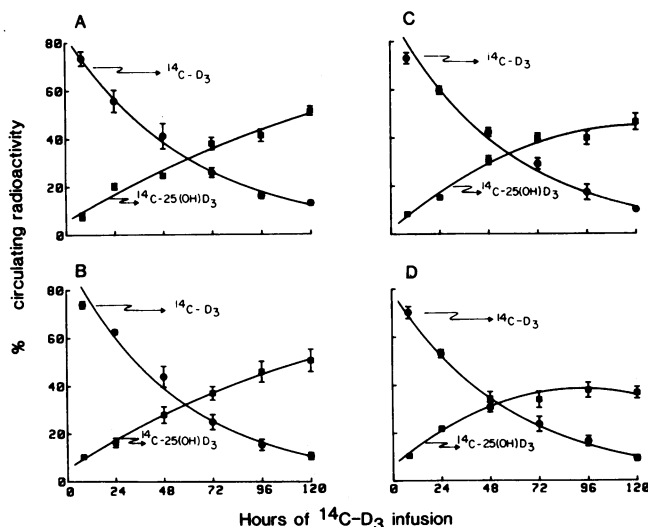

Figure 3. Qualitative chronological evolution of plasma radioactivity in calcium- and hormone-supplemented vitamin D–depleted rats during constant intraperitoneal infusion of [^{14}C]D$_3$. Time 0 h corresponds to 10 d of oral calcium gluconate supplementation (*B*) and one week of constant infusion with vehicle (controls, *A*) or doses of 7 (*C*) or 65 pmol 1,25(OH)$_2$D$_3$·24 h^{-1} (*D*). All supplementation regimens were continued during [^{14}C]D$_3$ infusion. The percentage of circulating radioactivity in the form of [^{14}C]D$_3$ (— ● —) followed a logarithmic decrease in time: (*A*) $\log y = -0.0070\ x + 1.92$, $r^2 = 0.992$; (*B*) $\log y = -0.0079\ x + 1.97$, $r^2 = 0.991$; (*C*) $\log y = -0.0076\ x + 1.96$, $r^2 = 0.988$; (*D*) $\log y = -0.0075\ x + 1.90$, $r^2 = 0.990$. The percentage of circulating radioactivity in the form of 25(OH)-[^{14}C]D$_3$ (— ■ —) was described by second order functions: (*A*) $y = -0.00097\ x^2 + 0.487\ x + 5.684$, $r^2 = 0.975$; (*B*) $y = -0.00124\ x^2 + 0.533\ x + 5.303$, $r^2 = 0.997$; (*C*) $y = -0.00289\ x^2 + 0.706\ x + 2.01$, $r^2 = 0.979$; (*D*) $y = -0.00371\ x^2 + 0.690\ x + 6.525$, $r^2 = 0.960$.

ministration but their plasma calcium concentration rose gradually over the following 72 h to reach normal values from 96 h on. This time point also corresponded to the stabilization of the circulating molar concentrations of [^{14}C]D$_3$. The plasma calcium concentrations of groups infused with 7 or 65 pmol 1,25(OH)$_2$D$_3$·24 h^{-1} were not significantly affected in time by [^{14}C]D$_3$ infusion. Both groups remained at their respective normal and slightly hypercalcemic levels. On the other hand, calcium-supplemented rats, which had reached normal plasma calcium concentrations prior to the onset of [^{14}C]D$_3$ infusion, responded to [^{14}C]D$_3$ administration by a gradual rise in plasma calcium to reach values equivalent to the 65-pmol 1,25(OH)$_2$D$_3$·24 h^{-1} group from 72 h of [^{14}C]D$_3$ infusion on.

Fig. 3 illustrates the kinetic behavior of the circulating [^{14}C]D$_3$ and 25(OH)-[^{14}C]D$_3$ in relation to total circulating ^{14}C activity. The percentage of plasma radioactivity in the form of [^{14}C]D$_3$ was seen to decrease logarithmically in all groups. On the other hand, the proportion of circulating radioactivity composed of 25(OH)-[^{14}C]D$_3$ increased in a manner best described by a second order function but with apparent differences between treatments. Indeed, 25(OH)-[^{14}C]D$_3$ in the vehicle-infused (Fig. 3 *A*) and calcium-supplemented (Fig. 3 *B*) groups occupied a gradually
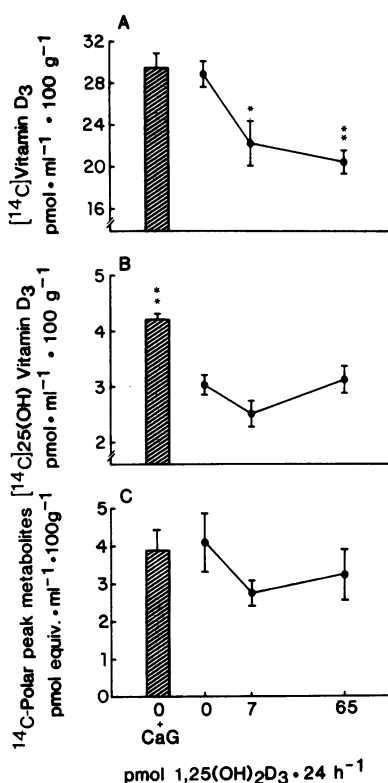

Figure 4. Plasma concentrations of vitamin D$_3$ and metabolites following 8 h of [^{14}C]D$_3$ administration in calcium- and hormone-supplementated rats (*A*) [^{14}C]D$_3$; (*B*) 25(OH)-[^{14}C]D$_3$; (*C*) [^{14}C]D$_3$ molar equivalents eluting under "polar peak" of the chromatogram. All rats received 2 μg [^{14}C]D$_3$ as a single intravenous injection and were infused intraperitoneally with 1.35 μg [^{14}C]D$_3$·24 h^{-1}, 8 h prior to this blood sample: this time-point also corresponded to 10 d of oral calcium gluconate supplementation (striped bars) and 1 wk of infusion with vehicle (o) or doses of 7 or 65 pmol 1,25(OH)$_2$D$_3$·24 h^{-1}. Results are expressed as means±SEM. $n = 26$ or six to seven rats per subgroup. Statistically significant differences between group means analyzed by ANOVA were different from control (o), *$P < 0.01$, **$P < 0.001$.

greater percentage of the circulating pool of radioactivity, which exceeded 50% following 120 h of $[^{14}C]D_3$ infusion, whereas in the $1,25(OH)_2D_3$-supplemented groups, a dose-dependent tendency to plateau at lower percentages from 72 and 48 h of $[^{14}C]D_3$ infusion on was observed for the 7 and 65 pmol $\cdot$ 24 h^{-1} groups, respectively (Fig. 3 C and D). The crossover between the $[^{14}C]D_3$ and $25(OH)-[^{14}C]D_3$ curves occurred at an earlier time point in the 65-pmol $1,25(OH)_2D_3 \cdot 24$ h^{-1} group than in the other groups (50.6±2.7 vs. 59.7±2.7, 58.6±3.8, and 57.6±2.3 h for the unsupplemented, calcium-supplemented, and 7-pmol $1,25(OH)_2D_3 \cdot 24$ h^{-1} groups, respectively, $P < 0.05$).

Figs. 4 and 5 present the plasma molar concentrations of $[^{14}C]D_3$ and $25(OH)-[^{14}C]D_3$, or of metabolites more polar than $25(OH)D_3$ ("polar peak") in the early phase (8 h after $[^{14}C]D_3$ administration, Fig. 4) and at a time point considered to represent steady state conditions (120 h after $[^{14}C]D_3$ administration, Fig. 5) since, by then, all groups displayed stable levels of total circulating radioactivity and molar concentrations of $[^{14}C]D_3$ and $25(OH)-[^{14}C]D_3$. Moreover, the hypocalcemic rats had normalized their plasma calcium while the plasma calcium concentrations of all other groups had reached stable levels from 72 h of $[^{14}C]D_3$ infusion on. As is illustrated in Fig. 4 A, oral calcium supplementation had no effect on the early circulating concentrations of unmetabolized $[^{14}C]D_3$. $[^{14}C]D_3$ concentrations were, however, significantly decreased in groups receiving 7 or 65 pmol $1,25(OH)_2D_3 \cdot 24$ h^{-1} in comparison to controls or calcium-supplemented animals. Fig. 4 B illustrates that the plasma concentrations of $25(OH)-[^{14}C]D_3$ were not significantly influenced by the infusion of exogenous $1,25(OH)_2D_3$, whereas calcium supplementation was associated with significantly higher circulating levels of $25(OH)-[^{14}C]D_3$ than controls or $1,25(OH)_2D_3$-infused animals. The circulating levels of polar peak metabolites (excluding dihydroxylated and more polar derivatives removed in the first column chromatographic step) (Fig.
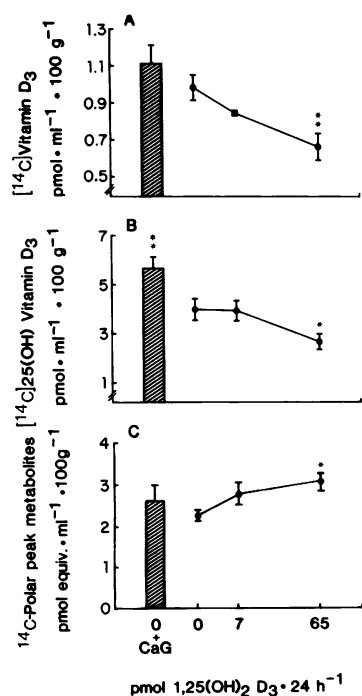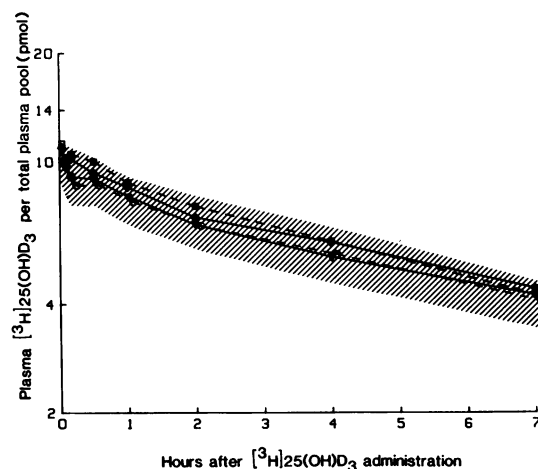

Figure 6. Plasma elimination of $25(OH)-[^3H]D_3$ in calcium- and hormone-supplemented vitamin D–repleted rats. All rats were injected with a single intravenous dose of 6 ng $25(OH)-[^3H]D_3$, 144 h after initiation of $[^{14}C]D_3$ infusion; this also corresponded to day 13 of continuing supplementation regimens. The elimination constants evaluated from the slopes of these lines (note the logarithmic scale on the ordinate) were 23.5±0.4, 23.0±0.6, 22.8±1.8, and 25.8±2.3 fmol $\cdot$ h^{-1} for the unsupplemented — ◇ — ($n = 5$), calcium-supplemented — ▼ — ($n = 5$), and 7 - - - ○ - - - ($n = 5$), or 65 pmol $1,25(OH)_2D_3 \cdot 24$ h^{-1} - - - □ - - - ($n = 4$) groups, respectively. The shaded area encompasses the standard error of all the slopes.

4 C) were not significantly affected, at this early time point, by either calcium or hormonal supplementation.

Fig. 5 A illustrates that, following 120 h of $[^{14}C]D_3$ infusion, all pretreatment regimens affected the circulating concentrations of unmetabolized $[^{14}C]D_3$ in a fashion similar to that observed following 8 h of $[^{14}C]D_3$ infusion. Indeed, animals infused with 65 pmol $1,25(OH)_2D_3 \cdot 24$ h^{-1} were associated with lower plasma $[^{14}C]D_3$ concentrations than controls, while oral calcium supplementation still had an insignificant effect. On the other hand, supplementation with 65 pmol $1,25(OH)_2D_3 \cdot 24$ h^{-1} led to plasma concentrations of $25(OH)-[^{14}C]D_3$ significantly lower than control while supplementation with 7 pmol $1,25(OH)_2D_3 \cdot 24$ h^{-1} had no effect (Fig. 5 B); oral calcium supplementation was again associated with significantly higher concentrations of circulating $25(OH)-[^{14}C]D_3$ than controls or $1,25(OH)_2D_3$-infused animals. Circulating levels of "polar peak" metabolites (Fig. 5 C) were not affected by oral calcium supplementation; however, they were gradually increased by exogenous hormone infusion to achieve values significantly greater than control at the 65 pmol $1,25(OH)_2D_3 \cdot 24$ h^{-1} dose.

Fig. 6 shows the plasma disappearance curves obtained following a single intravenous dose of exogenous $25(OH)-[^3H]D_3$. As estimated from the slopes of the curves, the $25(OH)-[^3H]D_3$ plasma elimination constants were similar in all groups. Dilution of the $25(OH)-[^3H]D_3$ by the $25(OH)-[^{14}C]D_3$ produced in vivo was evaluated in all treatment groups and found to have no influence on the elimination constant of the circulating $25(OH)-[^3H]D_3$.

Radioactivity derived from $[^{14}C]D_3$, which had accumulated in various tissues and organs at the end of the study is presented in Table I. The supplementation regimens did not affect the amount of radioactivity sequestered in the liver, adipose tissue (both brown and white), or skeletal muscle. On the other hand, the ^{14}C activity accumulated in the kidneys was significantly

Figure 5. Plasma concentrations of vitamin D_3 and metabolites following 120 h of $[^{14}C]D_3$ administration in calcium- and hormone-supplementated rats. (A) $[^{14}C]D_3$; (B) $25(OH)-[^{14}C]D_3$; (C) $[^{14}C]D_3$ molar equivalents eluting under "polar peak" of HPLC. All rats received 2 μg $[^{14}C]D_3$ as a single intravenous injection and were infused intraperitoneally with 1.35 μg $[^{14}C]D_3 \cdot 24$ h^{-1}, 120 h prior to this blood sample: this time point also corresponded to 10 d of oral calcium gluconate supplementation (striped bars), and 1 wk of infusion with vehicle (○) or doses of 7 or 65 pmol $1,25(OH)_2D_3 \cdot 24$ h^{-1}. Results are expressed as means±SEM. $n = 26$ or six to seven rats per subgroup. Statistically significant differences between group means analyzed by ANOVA were: different from control (○), $*P < 0.05$, $**P < 0.01$.

Table I. Tissue and Organ Accumulation of Circulating Radioactivity Derived from [^{14}C]Vitamin D_3*

Treatment group	n	Liver	Kidkeys	Adipose tissue		Skeletal Muscle
				Brown	White	
		pmol ^{14}C activity·total organ^{-1}·100 g body wt^{-1}		pmol ^{14}C activity·g tissue^{-1}·100 g body weight^{-1}		
Control	(6)	39.9±8.7	64.5±6.2	6.01±0.72	4.23±0.60	1.16±0.19
Calcium gluconate	(6)	45.7±8.8	54.2±8.9	8.51±1.74	3.80±0.81	1.43±0.51
7 pmol 1,25(OH)$_2$D$_3$·24 h^{-1}	(6)	40.4±5.8	57.4±5.3	6.31±0.72	3.68±0.70	1.26±0.36
65 pmol 1,25(OH)$_2$D$_3$·24 h^{-1}	(7)	32.6±4.2	13.4±2.5	5.71±0.61	3.22±0.31	0.67±0.13
ANOVA		NS	P < 0.001‡	NS	NS	NS

* Values are expressed as means±SEM and represent data corrected for contamination with plasma radioactivity (see Methods). ‡ Statistically significant difference was attributed to the 65-pmol 1,25(OH)$_2$D$_3$·day^{-1} group.

diminished by hormone infusion at the 65-pmol 1,25(OH)$_2$D$_3$·24 h^{-1} dose. The tissue accumulation of radioactivity derived from exogenous 25(OH)-[^{3}H]D$_3$ followed a pattern similar to that of ^{14}C, whether in the kidneys or in other tissues, and remained at levels below 1% of the injected dose in the liver and kidney, and below 0.1% in muscle and adipose tissue.

The molar concentrations of sequestered hepatic ^{14}C activity present in the form of D$_3$, 25(OH)D$_3$, or more polar metabolites are presented in Table II (top). Infusion with 65 pmol 1,25(OH)$_2$D$_3$·24 h^{-1} had a tendency to diminish the concentration of [^{14}C]D$_3$ and 25(OH)-[^{14}C]D$_3$ accumulated in the liver while calcium supplementation yielded an opposite effect, neither of which, however, achieved statistical significance. Similarly, supplementation regimens did not significantly influence the molar equivalent concentrations of more polar metabolites found sequestered in the liver. The radioactivity derived from exogenous 25(OH)-[^{3}H]D$_3$ was present as intact 25(OH)-[^{3}H]D$_3$ in a proportion of 50 to 57%, the remainder being associated with more polar derivatives; neither proportion was significantly affected by the supplementation regimens. Table II (bottom) presents the concentration ratios of 25(OH)-[^{14}C]D$_3$ to [^{14}C]D$_3$ as

well as those of more polar derivatives to 25(OH)-[^{14}C]D$_3$ as an indication of the intrahepatic metabolic activities. None of the ratios calculated were significantly affected by hormonal infusion or calcium supplementation.

Hepatic microsomal D_3-25 hydroxylase. The effects of in vivo calcium or hormonal supplementation on the in vitro assay of the hepatic microsomal D$_3$-25 hydroxylase activity are summarized in Table III. Microsomal cytochrome P-450 specific content was not significantly affected by the supplementation regimens. On the other hand, an overall significant effect of treatments on the D$_3$-25 hydroxylase activity was observed when the latter was expressed in terms of the microsomal protein present in the assay ($P < 0.05$). This effect was primarily attributed to a significant increase in enzyme activity in calcium-supplemented animals when compared with controls ($P < 0.01$). 1,25(OH)$_2$D$_3$ infusion also increased D$_3$-25 hydroxylase activity but the increase did not reach statistical significance. When the D$_3$-25 hydroxylase activity was expressed in terms of the cytochrome P-450 content (molar activity), a similar pattern of effects was obtained, albeit more discrete and thus statistically nonsignificant.

Table II. Hepatic [^{14}C]D_3 and 25(OH)-[^{14}C]D_3 Content

Treatment groups	n	D$_3$	25(OH)D$_3$	More polar metabolites
		pmol ^{14}C activity·total liver^{-1}·100 g body wt^{-1}		pmoleq ^{14}C activity·total liver^{-1}·100 g body wt^{-1}
Molar concentrations of ^{14}C activity present as intact D$_3$, 25(OH)D$_3$ or more polar metabolites*				
Control	(5)	22.0±5.9	7.43±1.43	13.9±3.2
Calcium gluconate	(5)	27.4±6.4	9.87±1.91	12.4±2.2
7 pmol 1,25(OH)$_2$D$_3$·24 h^{-1}	(5)	21.6±2.8	7.28±1.37	15.1±2.7
65 pmol 1,25(OH)$_2$D$_3$·24 h^{-1}	(4)	15.5±0.9	5.09±0.67	10.7±2.4
ANOVA		NS	NS	NS
Concentration ratios of accumulated ^{14}C-compounds‡				
Control	(5)		0.37±0.06	1.95±0.35
Calcium gluconate	(5)		0.40±0.06	1.27±0.10
7 pmol 1,25(OH)$_2$D$_3$·24 h^{-1}	(5)		0.35±0.06	2.18±0.27
65 pmol 1,25(OH)$_2$D$_3$·24 h^{-1}	(4)		0.33±0.04	2.15±0.54
ANOVA			NS	NS

* Individual concentrations were calculated using the percentage of total harvested radioactivity eluting under the respective portions of the chromatograms and results are expressed as means±SEM. [^{14}C]D$_3$ was infused over a period of 150 h. ‡ Values represent individual ratios of the respective compound concentrations and are expressed as means±SEM.

Table III. Hepatic Microsomal [^3H]D_3-25 Hydroxylase Activity

Treatment groups	n	Cytochrome P-450	Enzyme activity	
		nmol · mg protein^{-1}	*fmol 25(OH)-[^3H]D_3 produced · min^{-1} · 15 mg protein^{-1}*	*fmol 25(OH)-[^3H]D_3 produced · min^{-1} · nmol P-450^{-1}*
Control	(9)	0.515±0.016	5.52±0.61	0.688±0.069
Calcium gluconate	(10)	0.585±0.028	7.62±0.53*	0.839±0.048
7 pmol 1,25(OH)$_2$D$_3$ · 24 h^{-1}	(10)	0.567±0.029	6.62±0.43	0.801±0.040
65 pmol 1,25(OH)$_2$D$_3$ · 24 h^{-1}	(10)	0.520±0.024	6.36±0.43	0.809±0.071
ANOVA		NS	P < 0.05	NS

Orthogonal contrasts were tested according to the method of Dunnett (21). * Different from vitamin D-depleted hypocalcemic rats, P < 0.01.

Discussion

A subchronic in vivo protocol, using hypocalcemic vitamin D–depleted rats as the animal model, was designed in order to differentiate the effects of the hormone 1,25(OH)$_2$D$_3$ from those of plasma calcium normalization on the metabolism of D$_3$ to 25(OH)D$_3$. Constant intraperitoneal infusion with doses of 7 or 65 pmol 1,25(OH)$_2$D$_3$ · 24 h^{-1} for a period of 1 wk was followed by a dose-dependent increase in the plasma concentration of 1,25(OH)$_2$D$_3$ and total calcium whereas vehicle-infused controls exhibited low subphysiological circulating concentrations of both hormone and calcium. In addition, another group of animals was supplemented with calcium to achieve normal plasma calcium concentrations despite levels of plasma 1,25(OH)$_2$D$_3$ equivalent to those observed in unsupplemented animals. The plasma calcium normalization of this group may have been related in greater part to passive calcium transfer across the intestinal mucosa rather than to a 1,25(OH)$_2$D$_3$-stimulated absorption, as is suggested by the more sluggish nature of the calcemic response in comparison to that of the hormonally infused animals. This calcium-supplemented group enabled us to distinguish the effects of exogenous 1,25(OH)$_2$D$_3$ repletion from those associated with correction of the hypocalcemic state alone. The trophic effects on body and liver weights observed following hormone infusion, but not vehicle infusion or calcium supplementation, also confirmed the relevance and the physiological significance of the supplementation regimens. In the second part of the in vivo protocol, a constant infusion with physiological doses of [^{14}C]D$_3$ was initiated in all groups. As indicated by the plasma calcium concentrations of the unsupplemented controls, a gradual transition took place from the initial hypocalcemic vitamin D–depleted state to a stable normocalcemic state that was suggestive of a vitamin D–replete status. The normal physiologic action of the putative endogenously formed 1,25(OH)$_2$-[^{14}C]D$_3$ on the active intestinal calcium transport (and/or on other calcium mobilization processes) was probably responsible for this transition. On the other hand, the plasma calcium response of the calcium-supplemented animals, which gradually exhibited plasma calcium concentrations equivalent to those observed in the 65-pmol 1,25(OH)$_2$D$_3$ · 24 h^{-1} group, could have resulted from the limited production of endogenous 1,25(OH)$_2$-[^{14}C]D$_3$ in this group of animals which were still depleted in vitamin D at the onset of [^{14}C]D$_3$ infusion; indeed, small amounts of endogenously formed hormone may have been sufficient to increase plasma calcium in the presence of persistently elevated dietary calcium intake. By contrast, animals already receiving exogenous 1,25(OH)$_2$D$_3$ did not show any significant modifi-

cations of their plasma calcium concentrations during [^{14}C]D$_3$ infusion.

We thus examined the circulating parameters of [^{14}C]D$_3$ metabolism at the earliest sampling time when differences in vitamin D status between groups were still valid on the basis of plasma calcium values. Results showed that oral calcium supplementation was associated with significantly greater circulating concentrations of 25(OH)-[^{14}C]D$_3$ than controls despite equivalent plasma levels of substrate and other polar metabolites. This was further confirmed by the stimulatory effect of the oral calcium supplementation regimen on the in vitro microsomal D$_3$-25 hydroxylase activity. In contrast, 1,25(OH)$_2$D$_3$ infusion diminished the plasma concentrations of [^{14}C]D$_3$ without significantly affecting the circulating concentrations of its metabolites, and it did so in a manner independent of the dose administered. The presence of lower plasma [^{14}C]D$_3$ in the 1,25(OH)$_2$D$_3$ supplemented groups is suggestive of modifications in the early disposition of [^{14}C]D$_3$, which could be due to either increases in the storage of the vitamin in reserve sites or to increases in its excretion via the bile, urine, or pulmonary pathways. It is not excluded that the modifications in the early disposition of [^{14}C]D$_3$ could also be partly due to increases in the turnover of the vitamin as suggested by the data showing slightly increased in vitro D$_3$-25 hydroxylase activities.

The circulating parameters of [^{14}C]D$_3$ metabolism following 120 h of [^{14}C]D$_3$ infusion gave us more insight on the effects of exogenous 1,25(OH)$_2$D$_3$ administration and/or of the associated slight hypercalcemia on the long-term handling of [^{14}C]D$_3$. Indeed, all groups were vitamin D repleted by then and could thus have formed their own 1,25(OH)$_2$D$_3$. The data obtained at that time point show that oral calcium supplementation was still associated with an apparent stimulation of 25(OH)-[^{14}C]D$_3$ production. On the other hand, animals given the supplemental physiological dose of 7 pmol 1,25(OH)$_2$D$_3$ · 24 h^{-1} displayed plasma parameters of [^{14}C]D$_3$ metabolism essentially similar to those of unsupplemented controls. This observation is not surprising since unsupplemented animals probably had, by then, reached a vitamin D–replete status equivalent to that observed in the group receiving physiological amounts of 1,25(OH)$_2$D$_3$. After 120 h of constant [^{14}C]D$_3$ administration, however, rats infused with the pharmacological dose of 65 pmol 1,25(OH)$_2$D$_3$ · 24 h^{-1} were associated with significantly lower plasma concentrations of [^{14}C]D$_3$ and 25(OH)-[^{14}C]D$_3$ than controls while displaying significantly greater circulating concentrations of polar peak metabolites. These observations are suggestive of an accelerated metabolism of [^{14}C]D$_3$ and/or of

25(OH)-[^{14}C]D$_3$, and are well supported by the data on the kinetic behavior of the circulating ^{14}C activity in the 65-pmol 1,25(OH)$_2$D$_3 \cdot 24$ h^{-1} group. The studies on the plasma elimination kinetics of an exogenous dose of 25(OH)-[^{3}H]D$_3$, however, showed similar elimination constants in all groups indicating that the handling of 25(OH)D$_3$ is most likely not perturbed by oral calcium or 1,25(OH)$_2$D$_3$ supplementation. Moreover, the evaluation, after 120 h of [^{14}C]D$_3$ infusion, of the 25(OH)-[^{14}C]D$_3$/[^{14}C]D$_3$ plasma concentration ratio indicated similar ratios in all groups. These data collectively suggest that it might be the metabolism of D$_3$ rather than that of 25(OH)D$_3$ that is accelerated by 1,25(OH)$_2$D$_3$ infusion.

The data on the tissue accumulation of radioactivity derived from [^{14}C]D$_3$ did not show any significant effect of the supplementation regimens except in the kidney where 1,25(OH)$_2$D$_3$ infusion at the dose of 65 pmol $\cdot$ 24 h^{-1} greatly diminished the ^{14}C activity sequestered by the organ. These data thus suggest that the sequestration of D$_3$ or D$_3$-derived metabolites by known reserve sites such as the muscle and the adipose tissue was not affected by the supplementation regimens. The hepatic pattern of radioactivity also revealed similar plasma/liver [^{14}C]D$_3$ and 25(OH)-[^{14}C]D$_3$ concentration ratios in all groups indicating that the hepatic sequestration of D$_3$ and the export of 25(OH)$_3$ to the plasma were not perturbed by 1,25(OH)$_2$D$_3$ or calcium administration. Metabolites more polar than 25(OH)-[^{14}C]D$_3$ were, however, found in slightly lower concentrations in the liver than in the plasma of animals receiving the high 1,25(OH)$_2$D$_3$ dose as compared with the other groups, suggesting that these metabolites might have been formed in organs or tissues other than the liver.

Recent in vitro studies in the rachitic rat liver (12, 23) as well as in vivo experiments in man (13) have brought forward the observation of decreased 25(OH)D$_3$ concentrations when the substrate, vitamin D$_3$, and its hormonal metabolite, 1,25(OH)$_2$D$_3$, were introduced concomitantly. Both groups interpreted their data as indicative of an inhibitory effect of 1,25(OH)$_2$D$_3$ on the hepatic D$_3$-25-hydroxylase, and a scheme of regulation of hepatic vitamin D metabolism has been proposed on that basis (24). In the in vitro studies mentioned above (12, 23), however, D$_3$ together with supraphysiological or pharmacological doses of 1,25(OH)$_2$D$_3$ were introduced directly in vitro instead of conditioning the enzymatic processes involved in the production of 25(OH)D$_3$ by an in vivo pretreatment with the hormone as was done during the present studies. Studies on the vitamin D$_3$-25-hydroxylase in the presence of high doses of 1,25(OH)$_2$D$_3$ in the incubation media, or in a closed recirculating isolated perfused liver system, as was done by Baran and Milne (12, 23) may well lead to inhibition of the enzyme due to the mere presence of high doses of vitamin D sterols (1,25(OH)$_2$D$_3$ or further metabolites of the hormone) other than the natural substrate of the enzyme, vitamin D$_3$. On the other hand, Bell and co-workers (13) observed in their in vivo studies that there was no increase in the serum 25(OH)D concentrations in human volunteers supplemented with 2 μg $\cdot$ d^{-1} of 1,25(OH)$_2$D$_3$ for 4 d in association with 100,000 IU $\cdot$ d^{-1} of vitamin D as compared with that seen following supplementation with 100,000 IU $\cdot$ d^{-1} of vitamin D alone for 4 d. Interestingly, our in vivo protocol confirmed the observations made by Bell and co-workers (13) by showing reduced 25(OH)D$_3$ concentrations in animals receiving pharmacological doses of 1,25(OH)$_2$D$_3$; moreover, our results clearly showed, as pointed out by Bell et al. (24) in their study, that the effect of the hormone was independent of the

circulating plasma calcium concentrations. The experimental protocol used by Bell and co-workers, however, did not allow them to monitor the circulating concentrations of substrate or of other metabolites derived from the administered vitamin, which led them to conclude that 1,25(OH)$_2$D$_3$ inhibited the hepatic production of 25(OH)D. The data obtained during the present studies indicate that the handling of vitamin D in the presence of 1,25(OH)$_2$D$_3$ might be more complex than anticipated and do not generally support the concept of a physiologically meaningful inhibitory effect of 1,25(OH)$_2$D$_3$ on the hepatic vitamin D$_3$-25 hydroxylase. Indeed, as mentioned above, our results show a decrease in the circulating concentrations of 25(OH)D$_3$ as well as a decrease in the circulating concentrations of substrate, an increase in the plasma concentrations of metabolites other than 25(OH)D$_3$, similar intrahepatic and plasma ratios of 25(OH)D$_3$ to D$_3$ in all groups, and a lack of inhibitory effect of the 1,25(OH)$_2$D$_3$ pretreatment on the in vitro microsomal D$_3$-25 hydroxylase. In fact, the results of the present studies indicate that if 1,25(OH)$_2$D$_3$ pretreatment did influence hepatic vitamin D metabolism, its effect was rather modest and apparently not associated with an inhibition of the enzyme. Furthermore, the results of the present studies indicate that the plasma calcium concentrations, per se, seem to influence the hepatic production of 25(OH)D$_3$ both in vivo and in vitro.

Acknowledgments

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