25-Hydroxyvitamin D Metabolism in the Sheep Fetus and Lamb

SANG WHAY KOOH AND REINHOLD VIETH

Departments of Paediatrics and Physiology
University of Toronto and
The Research Institute, The Hospital for
Sick Children, Toronto, Canada.

SUMMARY

The metabolism of [^3H]-25-hydroxyvitamin D₃ (^3H -25-OH-D₃) to [^3H]-1,25-dihydroxyvitamin D₃ (^3H -1,25-(OH)₂D₃) and [^3H]-24,25-dihydroxyvitamin D₃ (^3H -24,25-(OH)₂D₃) was studied in sheep fetuses and lambs. *In vivo* metabolism was observed by chromatography of extracts of plasma obtained 24 hr after direct iv injection of ^3H -25-OH-D₃ to the fetus and lamb. The renal enzymes 25-hydroxyvitamin D-1-hydroxylase and -24-hydroxylase were determined *in vitro* by quantitative assay.

In fetal lambs, ^3H -24,25-(OH)₂D₃ was consistently the only detectable dihydroxyvitamin D₃ metabolite; this was confirmed by the *in vitro* studies in which 24-hydroxylase was the only detectable enzyme. Parathyroid hormone infusion to the fetus did not stimulate ^3H -1,25-(OH)₂D₃ production. In normal lambs fed sheep's milk or cow's milk ^3H -24,25-(OH)₂D₃ was the only metabolite of ^3H -25-OH-D₃ *in vivo* and only 24-hydroxylase activity was detected *in vitro*. In lambs fed a low-calcium, vitamin D-free formula, 24-hydroxylase activity remained measurable for at least 11 days of feeding, and by that time 1-OHase activity became evident *in vitro*. Appreciable ^3H -1,25-(OH)₂D₃ was not observed in plasma until 16 days of feeding with the formula. After 18 days on low-calcium, vitamin D-free formula, 1-OHase was the only detectable 25-OH-D₃ metabolizing enzyme. We also studied the rates at which ^3H -25-OH-D₃, ^3H -24,25-(OH)₂D₃ and ^3H -1,25-(OH)₂D₃ disappeared from the lamb plasma compartment. Within 1 hr, ^3H -1,25-(OH)₂D₃ fell to 50% of its initial concentration, and after 72 hr only 2% was left. In the case of ^3H -25-OH-D₃ and ^3H -24,25-(OH)₂D₃, 50% of each metabolite was left after 7 to 10 hr and about 20% after 72 hr. The rapid disappearance of ^3H -1,25-(OH)₂D₃ may explain the low levels or absence of this metabolite observed in the plasma of some animals shown to have renal 1-hydroxylase activity.

SPECULATION

Since 24,25-(OH)₂D₃ was the only detectable dihydroxyvitamin D₃ in the normal sheep fetus, this metabolite may be important for maintenance of calcium homeostasis and promotion of bone growth in health. Perhaps 1,25-(OH)₂D₃ is produced only when calcium homeostasis is disturbed by a deficiency of calcium and/or vitamin D.

INTRODUCTION

Recent studies indicate that 25-hydroxyvitamin D (25-OH-D) and 24,25-dihydroxyvitamin D (24,25-(OH)₂D) readily cross the placenta in rats and man from mother to fetus (8,9,10,15), but that very little 1,25-dihydroxyvitamin D (1,25-(OH)₂D) is thus transported in rats, the only species so far studied (15). Metabolism of 25-OH-D by the fetus is poorly understood. It has been reported that, when vitamin D-deficient pregnant rats were injected with ^3H -25-OH-D₃, ^3H -1,25-(OH)₂D₃ could be found in maternal and fetal tissue (7,13,23). The source of ^3H -1,25-(OH)₂D₃ recovered in the fetal tissue has not been clarified.

We sought direct evidence of the synthesis of 1,25-(OH)₂D or 24,25-(OH)₂D by the sheep fetus and newborn lamb by applying two independent measures of the animal's capacity to convert 25-OH-D to dihydroxyvitamin D. Firstly, we examined the conversion of 25-OH-D to 1,25-(OH)₂D after injecting radioactive 25-OH-D₃ directly into fetuses or newborn animals. Secondly, we evaluated the capacity of renal tissue from fetuses and young lambs to hydroxylate 25-OH-D by determining 25-hydroxyvitamin D-1-hydroxylase (1-OHase) and 24-hydroxylase (24-OHase) activities *in vitro* using a newly developed quantitative assay (21).

MATERIALS AND METHODS

FETUSES

Fetuses of Suffolk Cross ewes were studied *in situ*. Gestational age ranged from 131 to 143 days (normal gestation = 146 days). The ewes were raised on the Institute's experimental farm and were fed their usual diet of hay, corn grains, and molasses. They were brought to the Animal Facility of the Hospital for Sick Children several days before the experiment. Under halothane anesthesia, partial cesarian section was performed and the carotid artery and external jugular vein of the fetus (or one of twins) were cannulated (4); the venous cannula was for injection into the fetus and the arterial cannula was for blood sampling. Heparinized saline was infused continuously at a rate of 4 ml per 24 hr to maintain patency of the cannulas. We carried out subsequent experiments after the ewes had recovered completely from anesthesia, usually 24 to 48 hr after the operation.

IN VIVO 25-OH-D METABOLISM IN FETUSES

Four separate fetuses were used. Five μCi of [26,27- ^3H]-25-OH-D₃ (11.7 $\mu\text{Ci}/\text{mmol}$; Amersham Searle, Chicago, Ill) were injected through the venous catheter into two fetuses. Arterial blood (6-12 ml according to fetal size) was withdrawn 24 hr later and the plasma was submitted to lipid extraction (1) and chromatographed by the continuous-development TLC method of Vieth et al (21) to separate the vitamin D₃ metabolites. Metabolism was

assessed by measuring the percentage of total radioactivity in plasma that was recovered as ^3H -1,25-(OH)₂D₃ or ^3H -24,25-(OH)₂D₃. The latter two metabolites were identified by co-chromatography with authentic 1,25-(OH)₂D₃ or 24,25-(OH)₂D₃.

Bovine parathyroid hormone (Inolex Corp., Park Forest, South Ill) dissolved in 0.01 M acetic acid, was infused into the fetus of two ewes at the rate of 3.0 USP U/kg/hr for 24 hr. Five μCi ^3H -25-OH-D₃ were then injected intravenously. Arterial blood was withdrawn 24 hr later, extracted and chromatographed to separate the vitamin D₃ metabolites as indicated above.

IN VIVO 25-OH-D₃ METABOLISM IN LAMBS

a) Five lambs were delivered at term for this study. One lamb was studied at 2 days of age while it received ewe's milk. Another lamb was studied at 23 days of age while it fed cow's milk since 3 days of age. A third lamb was studied at 86 days of age, it had been weaned and was eating hay. The remaining two lambs were removed from their mothers at 3, and 19 days after birth, and fed a special low-Ca, vitamin D-free formula intended to stimulate 1-OHase activity. This formula consisted of 176 g strained lamb meat (Heinz), 22 g corn oil, and 88 g dextrose, mixed with water to a volume of 1 liter; each dl of the formula provided 67 Kcal and contained 1.0 mg Ca, 19.0 mg P, 1.9 g protein, 3.4 g fat, and 7.2 g CHO; it contained no vitamin D.

Ten μCi of ^3H -25-OH-D₃ were injected intravenously into lambs of 1 to 85 days of age. Twenty-four hr after injection, a blood sample was withdrawn and the plasma extracted and chromatographed to separate the vitamin D₃ metabolites as indicated above. Samples taken earlier than 24 hr after injection of ^3H -25-OH-D₃ gave qualitatively similar results but with somewhat smaller % yields of ^3H -dihydroxylated metabolites.

b) In another experiment, the effect of calcium and vitamin D deprivation on ^3H -25-OH-D₃ metabolism was investigated further in 2 pairs of lambs. In one pair the lambs were twins; in the other, the lambs were born at approximately the same time to different ewes. The smaller of each pair was suckled by the mother throughout the experiment; the other was fed the low-calcium, vitamin D-free formula starting in one animal at 3 days of age, and in another at 17 days of age.

Ten μCi of ^3H -25-OH-D₃ (9 $\mu\text{Ci}/\text{mmol}$) were injected to each lamb at the time of birth. Plasma samples were drawn serially up to 35 days after injection and lipid extracts of the samples were chromatographed.

c) An experiment to identify ^3H -dihydroxyvitamin D₃ metabolites found in plasma, intestine and kidney mitochondria was carried out in an additional lamb fed the low-Ca, vitamin D-free diet from 16 to 35 days of age. At 34 days of age, 10 μCi ^3H -25-OH-D₃ were injected intravenously, and 24 hr later the animal was exsanguinated under anesthesia. The kidneys were removed immediately and mitochondria prepared for study of 25-OH-D₃ metabolism *in vitro* as described below. Lipid extracts (1) of plasma, intestine (jejunum) and mitochondria were submitted to high-pressure liquid chromatography using a Waters Liquid Chromatograph fitted with a 6.2 mm x 25 cm Zorbax-Sil column. Development was with 80/10/10 hexane, methanol, methylene chloride at a flow rate of 2 ml per min. Fractions were analyzed by UV absorbance at 254 nm and radioactivity was measured in a Beckman Liquid Scintillation Counter.

ASSAY OF PLASMA 25-OH-D

Plasma 25-OH-D concentrations were measured by a binding-protein assay (11).

IN VITRO ASSAY OF 1-OHASE AND 24-OHASE ACTIVITIES IN FETUSES AND LAMBS

Enzyme determinations were made on the kidneys of 5 fetuses and 4 lambs. The fetuses were delivered by cesarian section and were exsanguinated before their kidneys were removed. The lambs were anesthetized with 300 mg Nembutal iv, they were then exsanguinated and the kidneys were removed.

Slices were taken from the outer 0.5 cm of the decapsulated kidney; 2-5 g of tissue were taken per pair of kidneys. The tissue was homogenized with a motor-driven Potter-Elvehjem homogenizer with 10 volumes of homogenization medium (250 mM sucrose, 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) buffer and 10 mM KCl, adjusted to pH 7.42 at room temperature). The preparation of kidney mitochondria and assay of the 25-OH-D hydroxylases was carried out according to the recently published method of Vieth and Fraser (21). All enzyme activities are the means of at least three replicate determinations.

DISAPPEARANCE RATES OF INJECTED ^3H -VITAMIN D METABOLITES IN LAMBS

The concentration of the ^3H -dihydroxy metabolite of vitamin D₃ in plasma represents a balance between the amount of metabolite produced by the kidney and the rate at which the metabolite is removed from the plasma compartment. To estimate the latter, we studied the disappearance rates of ^3H -25-OH-D₃, ^3H -24,25-(OH)₂D₃, and ^3H -1,25-(OH)₂D₃ from plasma after intravenous injection of each of these metabolites separately into two 4-month old lambs. Two μCi of metabolite were injected intrajugularly into each lamb. Six ml of blood were taken for analysis from the opposite jugular vein 10 min later. This value was taken as 100% of initial radioactivity. Thereafter 3 ml blood samples were obtained at several other times and subjected to extraction, chromatography and radioactive counting. From these results we prepared disappearance curves of the three substances.

RESULTS

³H-25-OH-D₃ METABOLISM IN FETUSES

The results of the study of ³H-25-OH-D₃ metabolism in sheep fetuses *in vivo* are shown in Table 1.

Treatment	None	None	PTH	PTH	Mean Maternal values
Gestation (days)	131	132	133	143	-
PLASMA VALUES					
Ca mg/dl	12.5	13.1	15.0	16.9	8.8
Pi mg/dl	6.5	9.2	10.2	9.6	6.0
25-OH-D ng/ml	12.7	8.6	-	3.1	26.0
% of ³ H*					
as 1,25-(OH) ₂ D ₃	nd+	nd	nd	nd	-
as 24,25-(OH) ₂ D ₃	1.2	7.4	5.4	12.0	-

* % of ³H in plasma that was recovered as ³H-1,25-(OH)₂D₃ or ³H-24,25-(OH)₂D₃. The blood was sampled 24 hr after iv injection of ³H-25-OH-D₃ and plasma was extracted and chromatographed.

+ not detectable, i.e. <0.2% of ³H on TLC.

Fetuses studied *in situ* had plasma calcium (Ca) and phosphorus (Pi) levels higher than those of the mothers. The chromatographic analysis of plasma extracts from the fetus showed ³H-24,25-(OH)₂D₃ as the only detectable metabolite. Only a trace of radioactivity was observed in maternal plasma; this was present as ³H-25-OH-D₃ indicating that the radioactive metabolites observed in the fetus originated in the fetus. PTH infusion into two fetuses raised the plasma Ca but did not decrease plasma Pi concentrations. PTH did not change the pattern of vitamin D metabolism.

Renal 24-OHase and 1-OHase activities in the sheep fetuses were consistent with the *in vivo* observations (Table 2). The only 25-OH-D metabolizing enzyme that could be detected was 24-OHase.

TABLE 2. ASSAY OF THE 25-OH-D-HYDROXYLASES IN FIVE NORMAL SHEEP FETUSES

Gestation (days)	1-OHase (pmoles/mg/min)*	24-OHase
105	nd+	nd
105	nd	0.025
135	nd	0.084
135	nd	0.065
135	nd	0.040

*pmoles 1,25-(OH)₂D₃ or 24,25-(OH)₂D₃ produced per mg mitochondrial protein per minute incubation.

+ not detectable: <0.01 pmoles (mg x min)⁻¹

³H-25-OH-D₃ METABOLISM IN LAMBS

Table 3 shows plasma Ca, Pi and 25-OH-D levels and *in vivo* metabolism of ³H-25-OH-D₃ in lambs fed various diets after birth. In lambs fed ewe's milk alone or fed ewe's milk followed by cow's milk or hay, ³H-24,25-(OH)₂D₃ was the only metabolite of ³H-25-OH-D₃. Lambs fed the low-Ca, vitamin D-free formula began to produce ³H-1,25-(OH)₂D₃ 8 days after starting the formula; by 16 days ³H-1,25-(OH)₂D₃ was the predominant ³H-dihydroxymetabolite.

Renal 24-OHase and 1-OHase activities in lambs are shown in Table 4. Lambs fed cow's milk or hay showed only 24-OHase activity. Lambs fed the low-Ca, vitamin D-free formula for 11 days had both 1-OHase and 24-OHase activities, whereas the lamb fed the formula for 18 days had only 1-OHase activity.

Figs. 1a and b show the results of chromatography of serial plasma samples taken from two pairs of lambs injected with ³H-25-OH-D₃ at birth. In both experiments, the lambs maintained on their mother's milk produced only ³H-24,25-(OH)₂D₃; the lambs fed the low-Ca, vitamin D-free diet showed sharply increased

TABLE 3. METABOLISM OF ³H-25-OH-D₃ IN LAMBS RECEIVING VARIOUS DIETS

Diet	ewe's milk	ewe's milk followed by cow's milk	ewe's milk followed by hay	low-Ca D-free+	low-Ca D-free
Days on diet	2	20	86	8	16
Age (days)	2	23	86	11	35
PLASMA VALUES					
Ca mg/dl	13.6	10.8	10.8	10.4	10.8
Pi mg/dl	12.8	11.4	7.8	7.2	7.1
25-OH-D ng/ml	3.0	5.2	12.4	<3.0	<3.0
% of ³ H*					
as 1,25-(OH) ₂ D ₃	nd	nd	nd	trace	1.5
as 24,25-(OH) ₂ D ₃	11.7	15.0	9.3	9.3	1.2

* % of ³H in plasma recovered as ³H-1,25-(OH)₂D₃ or ³H-24,25-(OH)₂D₃ 24 hr after iv injection of ³H-25-OH-D₃.

+ low-calcium, vitamin D-free formula.

TABLE 4. ASSAY OF 25-OH-D HYDROXYLASES IN FOUR LAMBS RECEIVING VARIOUS DIETS

Age	Feeding	1-OHase (pmoles/mg/min)*	24-OHase
26	cow's milk	nd+	0.031
180	hay	nd	0.023
14	4Ca D-free (for 11 days)	0.038	0.084
37	4Ca D-free (for 18 days)	0.053	nd

* pmoles 1,25-(OH)₂D₃ or 24,25-(OH)₂D₃ produced per mg mitochondrial protein per minute incubation.

+ not detectable: 0.01 pmoles (mg x min)⁻¹

In vivo studies were carried out in the latter two animals at 11 and 35 days of age (See Table 3).

Fig. 1. The effects of dietary deprivation of calcium and vitamin D on ³H-25-OH-D₃ metabolism (a) twins; (b) two lambs born at the same time to different mothers. ³H-25-OH-D₃ was injected at the time of birth. The low-Ca, vitamin D-free diet was started (+) to one lamb of each pair (.....); the other pair continued to suckle the mother (—). Values indicate relative amounts (%) of ³H-1,25-(OH)₂D₃ (●) and ³H-24,25-(OH)₂D₃ (○).

levels of ³H-24,25-(OH)₂D₃ in plasma after the special diet was introduced. The increases in ³H-24,25-(OH)₂D₃ were greater than could be accounted for by the increase in specific activity of substrate due to feeding the vitamin D-free diet. In the experiment shown in Fig. 1a, the lamb fed the low-Ca, vitamin D-free diet from 3 days of age died at 12 days of age, without detectable ³H-1,25-(OH)₂D₃ in the plasma. In the second experiment (Fig 1b), the lamb fed the low-Ca, vitamin D-free formula from 17 days of age had ³H-1,25-(OH)₂D₃ in plasma 17 days later. The kidneys from this lamb had abundant 1-OHase activity and showed no 24-OHase activity, whereas the milk-fed member of the pair had only 24-OHase activity.

The production of ³H-1,25-(OH)₂D₃ was verified in another lamb in which the same dietary manipulation was carried out and the metabolism of ³H-25-OH-D₃ was studied in greater detail. Chromatograms showing the patterns of radioactivity in extracts of plasma and intestine, and in the kidney mitochondrial enzyme assay are shown in Fig. 2. After 19 days of feeding with the low-Ca, vitamin D-deficient formula, the kidney mitochondria produced ³H-1,25-(OH)₂D₃ (Fig 2c). This metabolite was also present in the lipid extracts of plasma and intestine (Fig 2b and d). There was a significant amount of ³H-24,25-(OH)₂D₃ in the plasma, yet surprisingly there was little evidence of ³H-24,25-(OH)₂D₃ production in the renal mitochondrial incubation. This finding suggests the possibility of extrarenal synthesis of 24,25-(OH)₂D₃.

The rates of disappearance of ^3H -25-(OH) $_2\text{D}_3$, ^3H -1,25-(OH) $_2\text{D}_3$ and ^3H -24,25-(OH) $_2\text{D}_3$ from plasma following intravenous injections of synthetic metabolites into 4-month old lambs are shown in Fig. 3. ^3H -1,25-(OH) $_2\text{D}_3$ disappeared rapidly from plasma. Its initial plasma half-life was 1/2 hr, and after 3 days its level was 2% of the initial concentration. The initial plasma half-lives for ^3H -24,25-(OH) $_2\text{D}_3$ and ^3H -25-OH-D $_3$ were 7-10 hr, and the amounts remaining in plasma after 3 days were approximately 20%. The rapid disappearance of 1,25-(OH) $_2\text{D}_3$ from plasma helps to explain the low levels or absence of ^3H -1,25-(OH) $_2\text{D}_3$ observed in animals (Table 3) whose kidneys showed 1-OHase activity (Table 4 and Fig. 2).

Fig. 2. Radiochromatograms of tissue extracts from a lamb which had been fed the low-Ca, vitamin D-free diet for 19 days. (a) UV absorbance tracing from HPLC showing elution positions of authentic 24,25-(OH) $_2\text{D}_3$ and 1,25-(OH) $_2\text{D}_3$; (b) plasma; (c) kidney mitochondria incubated with ^3H -25-OH-D $_3$; (d) jejunum. The peak at fraction 13 is ^3H -25-OH-D $_3$.

Fig. 3. Disappearance of injected ^3H -vitamin D $_3$ metabolites from plasma. ^3H -25-OH-D $_3$ (●), ^3H -24,25-(OH) $_2\text{D}_3$ (▲) and ^3H -1,25-(OH) $_2\text{D}_3$ (■) were each injected separately into two lambs. After injection blood was drawn serially starting at 10 min (100% initial metabolite).

DISCUSSION

Our results show that the normal sheep fetus can synthesize ^3H -24,25-(OH) $_2\text{D}_3$ from ^3H -25-OH-D $_3$ and that 24-OHase is present in fetal kidney. However, the sheep fetus cannot synthesize ^3H -1,25-(OH) $_2\text{D}_3$ and there is no renal 1-OHase. Since the fetus is hypercalcemic and hyperphosphatemic, our findings are consistent with vitamin D metabolism under similar conditions in animals after birth where elevated calcium and phosphate suppress 1-hydroxylase and favor 24-hydroxylase activity (5). Since calcium and phosphate homeostasis of the fetus is maintained by placental transport and not by the fetal intestine and kidney, it is understandable that the fetus may have no need for 1,25-(OH) $_2\text{D}_3$, the most potent form of vitamin D.

Several studies have reported that 1,25-(OH) $_2\text{D}_3$ was detectable in the rat fetus (13, 23). These experiments differ from ours in many respects. Most importantly, the experimental mothers whose fetuses may have produced 1,25-(OH) $_2\text{D}_3$ had been fed a vitamin D-deficient diet; this is in contrast to our ewes which remained in their natural state and consumed normal diets. Furthermore, the evidence of others that 1,25-(OH) $_2\text{D}_3$ may have been produced by the fetus was indirect. We studied the metabolism of ^3H -25-OH-D $_3$ directly in intact fetuses as well as by their kidney mitochondria. Our finding that ^3H -24,25-(OH) $_2\text{D}_3$ is the most abundant dihydroxy metabolite of ^3H -25-OH-D in the fetus is consistent with findings in vitamin D-replete rats (15) and in the cord serum of humans (10).

We could not induce production of ^3H -1,25-(OH) $_2\text{D}_3$ by PTH infusion. The efficacy of the hormone infusion was indicated by the increased levels of plasma Ca. However, we do not have direct proof that PTH infusion had an effect on the renal handling of phosphate since there was no decrease in plasma phosphate in the fetuses infused with PTH; in addition we did not study phosphate clearance in these animals. Nonetheless, it may be safely assumed that the PTH infusion was effective on the kidneys of our sheep fetuses because other investigators have reported that PTH infusion in the sheep fetus decreases renal tubular reabsorption of phosphate, and that the renal effect is not always reflected in a decrease in plasma Pi (14, 17). It is conceivable that PTH infusion did not stimulate ^3H -1,25-(OH) $_2\text{D}_3$ production because the hormone infusion did not bring about adequate phosphate depletion to activate 1-OHase. Alternatively, 1,25-(OH) $_2\text{D}_3$ was not synthesized because there was no 1-OHase in the fetal kidney.

Two sets of investigations have offered a new concept to explain the presence of 1,25-(OH) $_2\text{D}_3$ in the fetus (13,23,24). They reported that nephrectomy in the pregnant rat did not prevent the appearance of ^3H -1,25-(OH) $_2\text{D}_3$ in maternal or fetal tissues after injection of ^3H -25-OH-D into the mother. These findings led to the hypothesis that the fetoplacental unit is the source of ^3H -1,25-(OH) $_2\text{D}_3$ production. Recently, Weisman et al (24) have reported that human decidua tissue in culture medium could synthesize 1,25-(OH) $_2\text{D}_3$. It is possible, therefore, that the ^3H -1,25-(OH) $_2\text{D}_3$ reportedly found in fetal rat tissue came from the placental unit.

The finding that 1,25-(OH) $_2\text{D}_3$ and 1-hydroxylase were consistently absent in normal lambs was unexpected. The theory that 1,25-(OH) $_2\text{D}_3$ is a hormone necessary for calcium homeostasis led us to anticipate that 1,25-(OH) $_2\text{D}_3$ would be present in the normal animals. Our results showed that young lambs were able to produce a significant amount of 1,25-(OH) $_2\text{D}_3$ only after prolonged feeding with the low-Ca, vitamin D-free diet. That the newborn lamb could not produce 1,25-(OH) $_2\text{D}_3$ quickly in response to a calcium and vitamin D-deficiency challenge may indicate that the newborn sheep has not fully developed the capacity to modulate 25-OH-D metabolism. Another implication of our studies is that 1,25-(OH) $_2\text{D}_3$ may not be essential for normal development of the growing animal, and that 1,25-(OH) $_2\text{D}_3$ is produced only when there is a perturbation of calcium homeostasis from such causes as inadequate supply of calcium or vitamin D. The relatively rapid disappearance of 1,25-(OH) $_2\text{D}_3$ from plasma is appropriate for a metabolite whose function is to correct disturbances in calcium homeostasis brought about by deficiency of calcium or vitamin D.

Our studies do not indicate which other vitamin D metabolites promote intestinal calcium absorption and maintain normal bone mineral metabolism if 1,25-(OH) $_2\text{D}_3$ is either absent or not required in growing animals. Can 24,25-(OH) $_2\text{D}_3$ fulfill these roles? We can only speculate at this time. Until recently 24,25-(OH) $_2\text{D}_3$ was considered to have little physiological action. Although not universally accepted (18), there is increasing evidence to suggest that 24,25-(OH) $_2\text{D}_3$ may be an essential metabolite (3,6,16,19). Kanis et al (12) reported that in both nephrectomized and normal humans, 24,25-(OH) $_2\text{D}_3$ increased intestinal calcium absorption almost as effectively as 1,25-(OH) $_2\text{D}_3$, but without increasing plasma Ca and urinary excretion. These authors postulated that the absorbed calcium entered the skeleton. Other workers have reported that 24,25-(OH) $_2\text{D}_3$ stimulated sulfate incorporation on growth plate chondrocytes (3), and that it was required for bone mineralization (2). Based on our data on the disappearance of labelled metabolites, the plasma turnover of 24,25-(OH) $_2\text{D}_3$ is much slower than that of 1,25-(OH) $_2\text{D}_3$. The relatively slower turnover rate of 24,25-(OH) $_2\text{D}_3$ is appropriate for a metabolite that regulates long-term skeletal metabolism; its blood level would not be expected to fluctuate rapidly.

REFERENCES

1. Bligh, E.G., Dyer, W.J.: A rapid method of total liquid extraction and purification. *Can J Biochem Physiol* 37: 911 (1959).
2. Bordier, P., Rasmussen, H., Marie, P., Miranet, L., Gueris, J., and Pyckwaert, A.: Vitamin D metabolites and Bone mineralization in man. *J Clin Endocrinol Metab* 46: 284 (1978).

3. Corvol, M.T., Dumontier, M.F., Garabedian, M. and Rappaport, R.: Vitamin D and cartilage. II Biological activity of 25-hydroxycholecalciferol and 24,25- and 1,25-dihydroxycholecalciferol on cultured growth plate chondrocytes. *Endocrinology* 102: 1269 (1978).
4. Dawes, G.S., Fox, H.E., Leduc, B.M., Liggins, G.C. and Richards, R.T.: Respiratory movements and rapid eye movement during sleep in the foetal lamb. *J Physiol* 220: 119 (1972).
5. DeLuca, H.F., and Schnoes, H.K.: Metabolism and mechanism of action of vitamin D. *Ann. Rev. Biochem* 45, 631 (1976).
6. Edelstein, S., and Ornoy, A.: 24,25-Dihydroxyvitamin D: the preferred metabolite for bone. Abstract, Fourth Workshop on Vitamin D, Berlin, Germany p. 99 (1979).
7. Gray, T.K., Lester G.E., and Lorenc, R.S.: Evidence for extra-renal 1 α -hydroxylation of 25-hydroxyvitamin D₃ in pregnancy. *Science* 204: 1311 (1979).
8. Haddad, J.G., Jr., Boisseau, V., and Avioli, L.: Placental transfer of vitamin D₃ and 25-hydroxycholecalciferol in the rat. *J. Lab Clin Med* 72: 908 (1971).
9. Hillman, L.S., and Haddad, J.G.: Human perinatal vitamin D metabolism 25-hydroxyvitamin D in maternal and cord blood. *J. Pediatr* 84: 742 (1974).
10. Hillman, L.S., Slatopolsky, E., Haddad, J.G.: Perinatal vitamin D metabolism IV. Maternal and cord serum 24,25-dihydroxyvitamin D concentrations. *J Clin Endocrinol Metab* 47: 1073 (1978).
11. Jones, G.: Assay of vitamin D₂ and D₃ and 25-hydroxyvitamin D₂ and D₃ in human plasma by high pressure liquid chromatography. *Clin Chem* 24: 287 (1978).
12. Kanis, J.A., Cundy, T., Bartlett, M., Smith, R., Heynen, G., Warner, G.T., and Russell, R.G.G.: Is 24,25-dihydroxycholecalciferol a calcium-regulating hormone in man? *Br Med J* 1: 1382 (1978).
13. Lester, G.E., Gray, T.K., and Lorenc, R.S.: Evidence for maternal and fetal differences in vitamin D metabolism. *Proc Soc Exp Biol Med* 159: 303 (1978).
14. Moore, E., Chung, E.E., Cevallos, E.E. and McMann, B.J.: Renal phosphate clearance in fetal lambs. *Pediat Res* 12: 1066 (1978).
15. Noff, D., and Edelstein, S.: Vitamin D and its hydroxylated metabolites in the rat. Placental and lacteal transport, subsequent metabolic pathways and tissue distribution. *Hormone Res* 9: 292 (1978).
16. Rasmussen, H., Bordier, P.: Vitamin D and bone. *Metab Bone Dis & Rel Res* 1: 7 (1978).
17. Smith, F.G., Tinglof, B.O., Meuli, J. and Borden, M.: Fetal response to parathyroid hormone in sheep. *J Appl Physiol* 27: 276, 1969.
18. Tanaka, Y., DeLuca, H.F., Kabayashi, Y., Tozuchi, T., Ikekawa, N. and Morisaki, M.: Biological activity of 24,24-difluoro-25-hydroxyvitamin D₃. Effect of blocking of 24-hydroxylation on the functions of vitamin D. *J Biol Chem* 254: 7163 (1979).
19. Thomasset, M., Redel, J., Mache, P., and Cuisinier-Gleizes, P.: Biological activity of 24R, 25- and 24S, 25-dihydroxycholecalciferol (24R, 25 and 24S, 25-(OH)₂D₃) on duodenal calcium-binding protein (d CaBP) and bone calcium mobilization in rats. In: A.W. Norman, K. Schaefer, J.W. Coburn, H.F. DeLuca, D. Fraser, H.G. Grigoleit, and D.V. Herrath. *Vitamin D, Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism* (Walter de Gruyter and Co., New York, p. 619, 1977).
20. Vieth, R., Fraser, D., and Jones, G.: Modified photography tank for continuous development of thin-layer chromatographic plates. *Anal Chem* 50: 2150 (1978).
21. Vieth R., and Fraser, D.: Kinetic behaviour of 25-hydroxyvitamin D-1-hydroxylase and -24-hydroxylase in rat kidney mitochondria. *J Biol Chem* 254, 12455, 1979.
22. Weisman, Y., Sapir, R., Harell, A., and Edelstein, A.: Maternal-perinatal interrelationships of vitamin D metabolism in rats. *Biochem Biophys Acta* 428: 388 (1976).
23. Weisman, Y., Vargas, A., Duckett, G., Reiter, E. and Root, A.W.: Synthesis of 1,25-dihydroxyvitamin-D in the nephrectomized pregnant rat. *Endocrinology* 103: 1992 (1978).
24. Weisman, Y., Harell, A., Edelstein, S., David, M., Spierer, Z. and Golanter, A.: 1 α ,25-dihydroxyvitamin D₃ and 24,25-dihydroxyvitamin D₃ in vitro synthesized by human decidua and placenta. *Nature* 281: 317 (1979).
25. We would like to thank Dr. Donald Fraser for advice and criticism and Mrs. Myrna Rawlins for her valuable technical assistance. Also thanks to Esther Rampersaud for her careful work in preparing this camera-ready manuscript.
26. Mailing address: Dr. S.W. Kooh
The Hospital for Sick Children
555 University Avenue
Toronto, Ontario M5G 1X8, Canada
27. This work was supported by a grant (MA 5139) from the Medical Research Council of Canada.
28. Received for publication September 17, 1979.
29. Accepted for publication January 11, 1980.