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# Evidence for Human Placental Synthesis of 24,25-Dihydroxyvitamin $D_3$ and 23,25-Dihydroxyvitamin $D_3$

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**ABSTRACT. The two principal dihydroxylated metabolites of the vitamin D prohormone 25-hydroxyvitamin $D_3$ [25(OH)$D_3$] are 1$\alpha$,25-dihydroxyvitamin $D_3$ [1,25(OH)$_2D_3$, the active hormone] and 24*R*,25-dihydroxyvitamin $D_3$ [24,25(OH)$_2D_3$, a putative regulator of developmental bone formation]. Although several studies have demonstrated placental synthesis of 1,25(OH)$_2D_3$ from 25(OH)$D_3$, placental production of 24,25(OH)$_2D_3$ has not been thoroughly investigated. Therefore, we studied 25(OH)$D_3$ metabolism in term human placenta using a villous explant model and cultures of isolated trophoblast and villous mesenchymal cells. We determined that both vitamin D-replete and vitamin D-deficient trophoblast metabolize 25(OH)$D_3$ predominantly via 24-hydroxylation. Placental 24,25(OH)$_2D_3$ was identified by cochromatography with authentic standard on four different HPLC systems, scanning UV spectrophotometry profile of the metabolite, sensitivity to periodate cleavage, and mass spectrometry of the putative placental 24,25(OH)$_2D_3$ and its periodate cleavage product. We also identified for the first time placental synthesis of 23,25(OH)$_2D_3$ using cochromatography with authentic standard on two different HPLC systems, scanning UV spectrophotometry, resistance to periodate cleavage, and mass spectrometry. When trophoblast was incubated for up to 4 h with physiologic concentrations of [$^3$H]25(OH)$D_3$ (6 nM) significant amounts of [$^3$H]24,25(OH)$_2D_3$ were produced, but [$^3$H]1,25(OH)$_2D_3$ could not be consistently detected. In contrast, when we incubated trophoblast with supraphysiologic concentrations of 25(OH)$D_3$ (6–10 $\mu$M), both 24,25(OH)$_2D_3$ and 1,25(OH)$_2D_3$ were synthesized. These results provide unequivocal evidence for placental synthesis of both 24,25(OH)$_2D_3$ and 23,25(OH)$_2D_3$. These findings also suggest that supraphysiologic substrate concentrations saturate the placental 24-hydroxylase and may permit accumulation of placental 1,25(OH)$_2D_3$ by preventing its further metabolism. Consequently, the identification of this high basal 24-hydroxylase activity in trophoblast may explain inconsistencies among previous reports regarding placental 1,25(OH)$_2D_3$ production. We speculate that active placental 24-hydroxylation may serve important functions in perinatal vitamin D metabolism. (*Pediatr Res* 34: 98–104, 1993)**

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Abbreviations

25(OH)$D_3$, 25-hydroxyvitamin $D_3$
1,25(OH)$_2D_3$, 1$\alpha$,25-dihydroxyvitamin $D_3$
24,25(OH)$_2D_3$, 24*R*,25-dihydroxyvitamin $D_3$
23,25(OH)$_2D_3$, 23*S*,25-dihydroxyvitamin $D_3$
26,25(OH)$_2D_3$, 26,25-dihydroxyvitamin $D_3$
DNase, deoxyribonuclease
DMEM, Dulbecco's Minimal Essential Medium
HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid

In birds and mammals, vitamin $D_3$ is 25-hydroxylated in the liver and converted to the prohormone 25(OH)$D_3$, which undergoes further hydroxylation steps principally in the kidney. The two major dihydroxylated metabolites produced in the kidney are 1,25(OH)$_2D_3$, which is the hormonal form of the vitamin (1), and 24,25(OH)$_2D_3$ (2, 3), a putative regulator of developmental bone formation (4–13). The biologic roles of the other known natural dihydroxylated vitamin $D_3$ metabolites, 23,25(OH)$_2D_3$ and 26,25(OH)$_2D_3$, are not well understood.

The observation that nephrectomized, vitamin $D_3$-deficient pregnant rats retain the ability to convert [$^3$H]25(OH)$D_3$ to [$^3$H]1,25(OH)$_2D_3$ *in vivo* (14, 15) raised the possibility that extrarenal sites produce 1,25(OH)$_2D_3$ during pregnancy. Subsequently, *in vitro* 1$\alpha$-hydroxylation of 25(OH)$D_3$ has been demonstrated in rat (14, 16), guinea pig (17) and human (18–22) placenta or decidua. In contrast to placental 1$\alpha$-hydroxylation, 24*R*-hydroxylation of 25(OH)$D_3$ has not been thoroughly investigated. Several reports have identified placenta as a possible site for 24-hydroxylation (16, 18–21), but none of these studies characterized the putative placental 24,25(OH)$_2D_3$ through more extensive chromatographic analysis or by mass spectrometry. Moreover, recently Hollis *et al.* (22) failed to demonstrate any 24,25(OH)$_2D_3$ production by either human placental homogenates or subcellular fractions, despite evidence for 1,25(OH)$_2D_3$ production in these preparations. These inconsistencies among previous reports prompted us to investigate whether placenta might possess a biologically important 25(OH)$D_3$-24-hydroxylation pathway. Like kidney, placenta apparently synthesizes 1,25(OH)$_2D_3$ and expresses 1,25(OH)$_2D_3$ receptors (23). In kidney, 24*R*- and 1$\alpha$-hydroxylase activities are regulated in a reciprocal fashion (24–26); 1,25(OH)$_2D_3$ generally suppresses its own synthesis and induces 24-hydroxylation by mechanisms that may require the presence of functional 1,25(OH)$_2D_3$ receptors (25, 27). Similarly, the elevated maternal plasma 1,25(OH)$_2D_3$ levels observed during pregnancy (28–31) [and 1,25(OH)$_2D_3$ produced by placenta itself] might be expected to stimulate placental 24-hydroxylation of vitamin $D_3$ metabolites.

In the present study, we examined human placental 25(OH)D$_3$ metabolism in several different culture systems. We present unequivocal evidence demonstrating that human placenta is a site for both 24,25(OH)$_2$D$_3$ and 23,25(OH)$_2$D$_3$ synthesis.

## MATERIALS AND METHODS

*Materials.* Cell and tissue culture media were obtained from Fisher Scientific (Malvern, PA). Except as noted, enzymes, cell culture reagents, and serum were from Sigma Chemical Co. (St. Louis, MO). Plasticware was purchased from Costar (Cambridge, MA). Crystalline 25(OH)D$_3$, 1,25(OH)$_2$D$_3$, 24,25(OH)$_2$D$_3$, and 23,25(OH)$_2$D$_3$ were generous gifts from Dr. Milan R. Uskokovic of Hoffman-LaRoche (Nutley, NJ). 25-hydroxy-[26,27-methyl-$^3$H]D$_3$ ([$^3$H]25(OH)D$_3$, 21 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). The purities of the tritiated and unlabeled vitamin D$_3$ metabolites were verified by HPLC before use. All HPLC solvents were "distilled in glass" spectroscopic grade from Burdick & Jackson Laboratories (Muskegan, MI).

*Placental cell and tissue (explant) isolation and culture.* Term human placentas were obtained at times of elective cesarean section in accordance with informed consent procedures approved by the Institutional Review Board of Women and Infants' Hospital of Rhode Island. Pregnancies complicated by disorders of mineral or vitamin D metabolism, hypertension or preeclampsia, preterm delivery, multiple gestation, or placental or fetal anomalies were excluded. All pregnant women received standard prenatal nutritional counseling and took a daily multivitamin preparation that included 400 IU of vitamin D$_3$. Placentas were transferred on ice and dissected under sterile conditions within 1 h. Decidua basalis was removed from the basal plate and discarded.

Cytotrophoblasts were isolated by the method of Kliman *et al.* (32) with modifications. Briefly, minced villous tissue was digested with 0.125% (wt/vol) trypsin and 0.02% DNase. A cytotrophoblast-enriched cell fraction was purified by sequential centrifugations through discontinuous Percoll density gradients. Cells isolated in this manner are ≥95% cytotrophoblasts gauged by morphologic and immunocytochemical criteria (33). When placed in culture, the mononuclear cytotrophoblasts aggregate, then fuse into syncytia approximately 72 h after plating. Cells were plated densely in either 35-mm cluster wells (5 to 7 × $10^6$ cells/well) or 60-mm culture dishes ($10^7$ cells/dish) and maintained in DMEM supplemented with 20 mM HEPES, 4 mM L-glutamine, antibiotics, and 15% (vol/vol) serum supplement (Nu-Serum IV, Collaborative Research, Bedford, MA). The concentration of fetal bovine serum in this complete medium is 3.75%. Cultures were refed with fresh medium every 24 h.

Placental fibroblasts were isolated by trypsin-DNase or collagenase-hyaluronidase-DNase (34) dispersion followed by differential adhesion in 75-cm$^2$ culture flasks. Fibroblasts were grown and serially passaged in DMEM supplemented with 4 mM L-glutamine, antibiotics, and 10% fetal bovine serum. Studies of 25(OH)D$_3$ metabolism were performed upon confluent fibroblast monolayers grown in 60-mm culture dishes (third to fifth passages).

Fragments of placental villi measuring ≤2 mm$^2$ (placental explants) were dissected from minced placental tissue and washed extensively in warm Hanks' buffered salt solution. Explants (≤1 g minced villi/100-mm plastic dish) were incubated in serum-free DMEM. Medium was changed daily for 1 to 4 d. Tissue viability during this period was demonstrated by maintenance of regulated expression and secretion of chorionic gonadotropin (Rubin LP, unpublished data).

*Incubations.* The assays of 25(OH)D$_3$ metabolism were carried out by incubating cells or tissue with graded concentrations of [$^3$H]25(OH)D$_3$ or unlabeled 25(OH)D$_3$ or both for various time intervals at 37°C. Short-term cell and tissue incubations (5 min to 4 h) were performed using MEM with Eagle's salts, 20 mM HEPES, and 0.1% BSA in a shaker water bath. Longer incubations (20 h) were performed using DMEM with 0.1% BSA on a rocking platform in a humidified 5% $CO_2$/95% air atmosphere. 25(OH)D$_3$ was dissolved in ethanol and added to incubation media to a final concentration of ≤0.1% (vol/vol). At the end of each incubation, cells or explants and media were collected together and added to one volume of methanol that contained authentic vitamin D$_3$ metabolites for HPLC standardization and assessment of extraction efficiency. Explants were homogenized by using three to four bursts from a Polytron tissue grinder.

*Lipid extraction.* Lipid extraction was based on the procedure of Bligh and Dyer (35), except that methylene chloride was substituted for chloroform. Lipid was extracted in a separatory funnel and the organic phase was reduced to dryness under a stream of nitrogen. Samples were redissolved in mobile-phase solvent and subjected to analytic or preparative liquid chromatography in 200-μL aliquots.

*Chromatography.* HPLC was performed on a Waters model 600 liquid chromatograph equipped with a model 990 photodiode array detector (Waters Associates, Milford, MA). All analytical and semipreparative microparticulate silica gel straight-phase columns (4.6 mm × 25 cm) were purchased from Dupont Instruments (Wilmington, DE). Flow rate in all instances was 2 mL/min. Various radioactive vitamin D$_3$ metabolites were identified by their retention times, determined by cochromatography with unlabeled authentic standards. Timed elution fractions were collected and counted by liquid scintillography, and radioactivity in each peak was calculated. Additionally, in some experiments using micromolar concentrations of 25(OH)D$_3$, metabolism of unlabeled 25(OH)D$_3$ was also monitored by scanning spectrophotometry of the purified peaks for their UV (205–320 nm) profiles.

Metabolism of [$^3$H]25(OH)D$_3$ to [$^3$H]1,25(OH)$_2$D$_3$ by placental cells or explants was initially analyzed by chromatography of the lipid extracts on a Zorbax-SIL column equilibrated with a 6% isopropanol:hexane solvent system. A peak of [$^3$H] radioactivity coeluting with the 1,25(OH)$_2$D$_3$ standard, obtained from the first HPLC system, was also analyzed by HPLC on a Zorbax-SIL column with a 3% isopropanol:methylene chloride solvent system and on a Zorbax-CN column with 4% isopropanol:hexane.

Production of [$^3$H]24,25(OH)$_2$D$_3$ was initially analyzed using a Zorbax-SIL column with a 2% isopropanol:hexane solvent system. Aliquots of the apparent 24,25(OH)$_2$D$_3$ peak were subjected to further chromatographic analysis and purification using *1*) a Zorbax-SIL column with 2% isopropanol:methylene chloride, *2*) a Zorbax-SIL column with 5% isopropanol:hexane, *3*) a Zorbax-CN column with 3% isopropanol:hexane, and *4*) an amino column with 2% isopropanol:hexane.

Production of 23,25(OH)$_2$D$_3$ also was initially analyzed chromatographically on a Zorbax-SIL column with 2% isopropanol:hexane. Further purification was achieved using a 1% isopropanol:methylene chloride solvent system. Purified 23,25(OH)$_2$D$_3$ and 24,25(OH)$_2$D$_3$ peaks were dried, resuspended, and analyzed by mass spectrometry.

*Periodate cleavage.* Putative 24,25(OH)$_2$D$_3$ or 23,25(OH)$_2$D$_3$ was subjected to periodate cleavage (36) by adding 10 μL of 5% aqueous sodium metaperiodate to 0.5 μg of purified sample or standard dissolved in 15 μL of methanol. Reactions were carried out for 30 min at room temperature. The soluble material was subjected to chromatography on a Zorbax-SIL column eluted with 2% isopropanol:hexane. Purified periodate cleavage product peaks were dried, resuspended, and analyzed by mass spectrometry.

*Mass spectrometry.* Material derived from HPLC-purified peaks with the scanning UV trace characteristics $\lambda_{max}$ = 265 nm, $\lambda_{min}$ = 228 nm was subjected to electron impact mass spectrometry using a VG-70SE double-focusing mass spectrometer with an E/B configuration (VG Analytical, Manchester, UK) and fitted with a direct insertion probe. Samples (~0.5 μg) in hexane:isopropanol:methanol (24/5/1) were introduced, and the

probe was heated from 30°C to 350°C over 30 min. Ionization voltage was 70 eV. Ions emerged at approximately 10 to 15 min after probe heating commenced.

*Statistical analysis.* Comparisons of production of 25(OH)$D_3$ metabolites were performed using the Wilcoxon signed-rank test. $p < 0.05$ was considered significant.

## RESULTS

*Time-course studies using physiologic concentrations of 25(OH)$D_3$ (6 nM).* When vitamin D-depleted trophoblast or villous explant cultures were incubated with 6 nM [$^3$H] 25(OH)$D_3$, there was detectable synthesis of a metabolite that comigrated with 24,25(OH)$_2D_3$ on Zorbax-SIL chromatography (Fig. 1*A*). Preincubation of trophoblast or explants with 1,25(OH)$_2D_3$ increased the conversion of [$^3$H]25(OH)$D_3$ to [$^3$H] 24,25(OH)$_2D_3$ from 8.2 to 13.1% of applied radioactivity (Table 1). During time-course experiments extending to 4 h, a peak of radioactivity frequently eluted in the expected position of 1,25(OH)$_2D_3$. As shown in Figure 1*A*, another peak, which emerged consistently within 1 to 2 min before the expected position of 1,25(OH)$_2D_3$, was distinguishable as a different compound by its slightly different retention time compared with the authentic 1,25(OH)$_2D_3$ internal standard. Based upon its chromatographic behavior in the hexane-based HPLC solvent system (37, 38), this placental metabolite may represent 19-nor-10-keto-25(OH)$D_3$. No significant metabolism of [$^3$H]25(OH)$D_3$ was detected using cultured placental mesenchymal core cells (fibroblasts) (Fig. 1*B*).

*Incubation of trophoblast and villous explants with supraphysiologic concentrations of 25(OH)$D_3$ (6–10 μM).* To produce μg quantities of the apparent placental dihydroxylated vitamin $D_3$ metabolites for further analysis, trophoblast or villous explants were incubated for 20 h in the presence of micromolar concentrations of 25(OH)$D_3$. Figure 2 shows chromatographic characterization of [$^3$H]1,25(OH)$_2D_3$ produced by incubating syncytiotrophoblast with 6 μM 25(OH)$D_3$ including 8 μCi of [$^3$H] 25(OH)$D_3$. Placental production of a metabolite comigrating with authentic 1,25(OH)$_2D_3$ was identified using three different HPLC systems. Because trophoblast and villous explant cultures produced nearly identical 25(OH)$D_3$ metabolic profiles, we used villous explants to produce placental vitamin $D_3$ metabolites in a quantity sufficient for their structural identification. Villous explants were incubated with 10 μM 25(OH)$D_3$ including 4 μCi of [$^3$H]25(OH)$D_3$. Chromatography of the placental lipid extract is shown in Figure 3. Using this 2% isopropanol:hexane solvent system, only two 25(OH)$D_3$ metabolite peaks, which eluted in the positions of 23,25(OH)$_2D_3$ and 24,25(OH)$_2D_3$, respectively (middle panel), exhibited UV absorbance spectra with a $\lambda_{max}$ at 265 nm and a $\lambda_{min}$ at 228 nm (top panel), consistent with relatively pure compounds containing the vitamin D *cis*-5,7,10(19)-triene chromophore. The bottom panel shows that the tritiated substrate was metabolized predominantly to [$^3$H] 23,25(OH)$_2D_3$ and [$^3$H]24,25(OH)$_2D_3$. Further attention was focused on these probable 24,25(OH)$_2D_3$ and 23,25(OH)$_2D_3$ peaks.

*Identification of placental 24,25(OH)$_2D_3$.* The putative 24,25(OH)$_2D_3$ peak was subjected to further preparative chromatography using a methylene chloride-based solvent system, then subjected to periodate cleavage. Both the native metabolite and its periodate cleavage product eluted as single peaks with the expected retention times (Fig. 4) and were then subjected to mass spectrometry. Injection of approximately 0.5 μg of placental 24,25(OH)$_2D_3$ emitted a molecular ion at m/z 416 and characteristic fragment peaks similar to the mass spectrum reported by Holick *et al.* (39) (data not shown). The mass spectrum of the 24,25(OH)$_2D_3$ periodate cleavage product (Fig. 5) showed a molecular ion at m/z 356, which results from cleavage of the $C_{24}$-$C_{25}$ bond yielding the corresponding 24-aldehyde. The fragmentation peaks at 323 (loss $H_2O$ and $CH_3$), 271 (side-chain cleavage), 253 (271-$H_2O$), 136 (*cis*-triene cleavage), and 118 (136-$H_2O$) mimic the published mass spectrum for this compound (39).

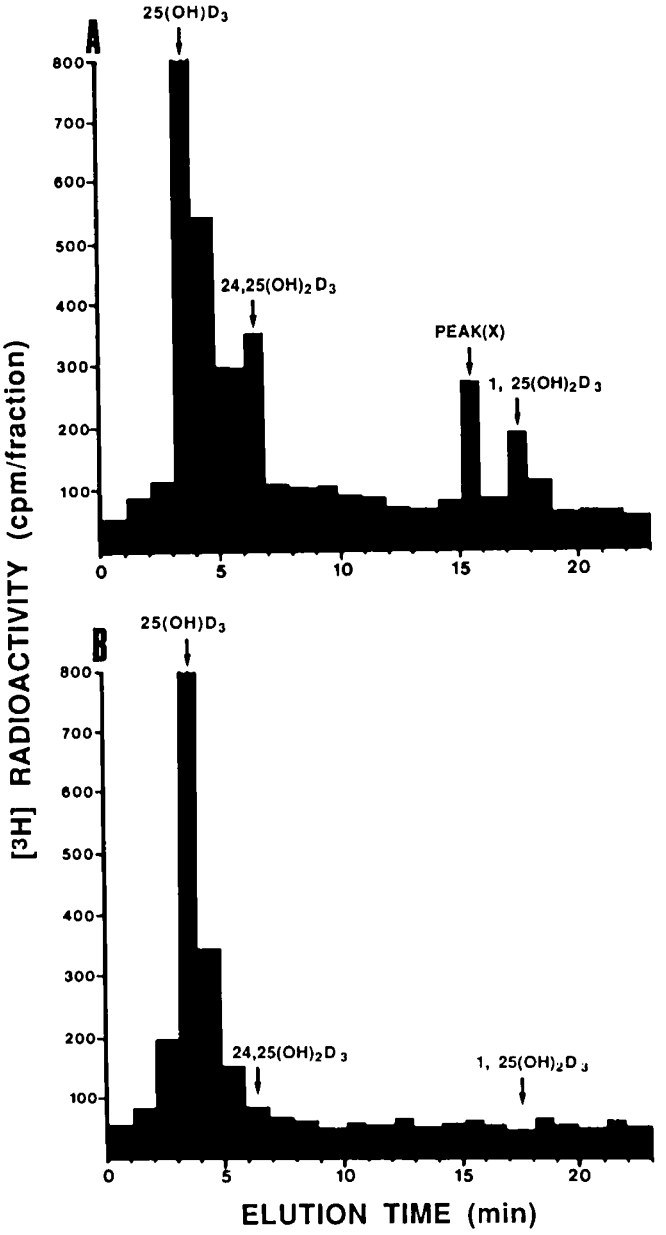


Fig. 1. Representative chromatograms of the lipid extracts (cells plus medium) of syncytiotrophoblast (*A*) and placental fibroblast (*B*) cultures. Cells were incubated for 4 h with 6 nM 25(OH)$D_3$ (including 40000 cpm/well of [$^3$H]25(OH)$D_3$). Recoveries were >90% of applied radioactivity. The HPLC system consisted of a Zorbax-SIL column equilibrated with 6% isopropanol:hexane. The peaks eluting in the positions of the purified 25(OH)$D_3$, 24,25(OH)$_2D_3$, and 1,25(OH)$_2D_3$ synthetic standards are so labeled. Peak *X* may correspond to 19-nor-10-keto-25(OH)$D_3$ (see text).

*Identification of placental 23,25(OH)$_2D_3$.* These placental 25(OH)$D_3$ incubations produced a second metabolite in a molar ratio of approximately 1:10 compared with 24,25(OH)$_2D_3$. This metabolite was further characterized as 23,25(OH)$_2D_3$ by HPLC using hexane- and methylene chloride-based solvent systems and by demonstrating the compound's resistance to periodate cleavage (data not shown). The mass spectrum of this placental 23,25(OH)$_2D_3$ peak (Fig. 6) revealed a molecular ion at m/z 416. Diagnostic fragments were emitted at 383 (loss $H_2O$ and $CH_3$), 253 (side-chain cleavage), 136, and 118, consistent with a dihy-

Table 1. *Effect of 1,25(OH)$_2$D$_3$ on production of [$^3$H]24,25(OH)$_2$D$_3$ by syncytiotrophoblast**

| Condition | Conversion [$^3$H]25(OH)$_2$D$_3$ to [$^3$H]24,25(OH)$_2$D$_3$ in 4 h (%) |
|---|---|
| Culture in vitamin D-deficient media for 72 h | 8.2 ± 0.5 |
| Preincubation with 50 nM 1,25(OH)$_2$D$_3$ for the final 16 h of culture | 13.1 ± 1.1† |

* Results are given as mean ± SEM; $n = 3$.

† $p < 0.01$ comparing trophoblast cultured with and without 1,25(OH)$_2$D$_3$ preincubation.

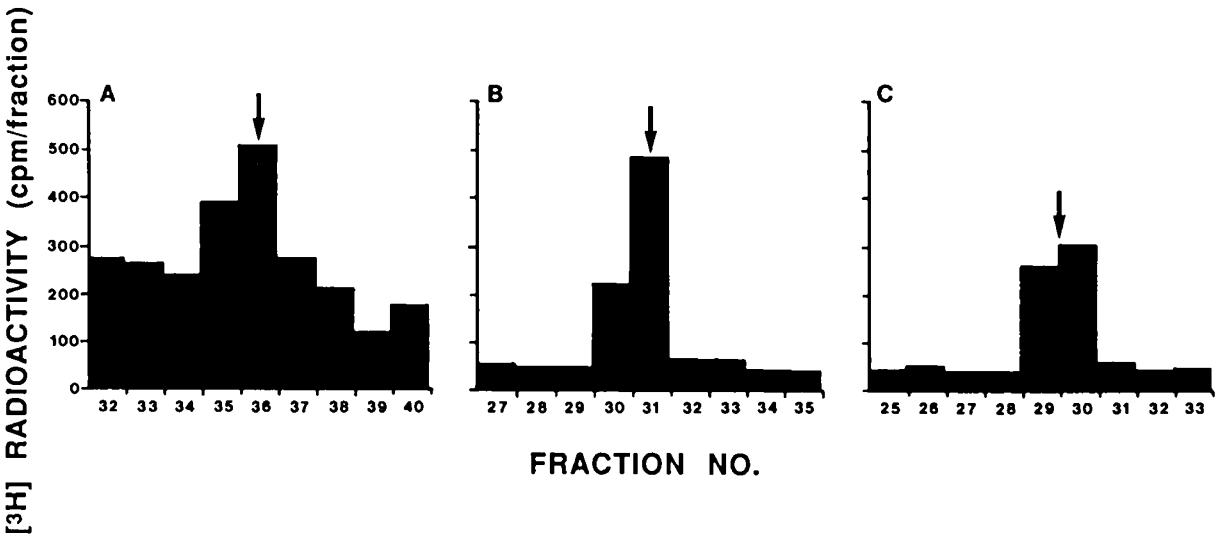


Fig. 2. Chromatographic detection of [$^3$H]1,25(OH)$_2$D$_3$ produced by cultured syncytiotrophoblast. Cultures were incubated with 6 μM 25(OH)D$_3$ (including 8 μCi of [$^3$H]1,25(OH)$_2$D$_3$). *Bars* represent the amount of tritiated material in eluent fractions obtained by analysis of the lipid extract (cells plus medium) on three different HPLC systems. One twentieth of the total $^3$H radioactivity was injected in each case: *A*, Zorbax-SIL column eluted with 6% isopropanol:hexane; *B*, Zorbax-SIL column eluted with 3% isopropanol:methylene chloride; *C*, cyanide column eluted with 4% isopropanol:hexane. *Arrows* indicate elution positions for the cochromatographed synthetic 1,25(OH)$_2$D$_3$ standard (detected by UV absorbance at 265 nm).

droxylated vitamin D$_3$ derivative possessing an intact triene. Distinctive 23,25(OH)$_2$D$_3$ mass spectrum fragmentation peaks (which are not observed with other dihydroxylated vitamin D$_3$ metabolites) were emitted at m/z 324 ($C_{23}$-$C_{24}$ cleavage with proton transfer from the larger to the smaller fragment and loss $H_2O$) and 309 ($C_{23}$-$C_{24}$ cleavage, proton transfer, and loss $CH_3$) (40). This placental metabolite mass spectrum is very similar to previously reported 23,25(OH)$_2$D$_3$ mass spectra (40–42).

## DISCUSSION

Placental 24,25(OH)$_2$D$_3$ synthesis was tentatively identified in several studies of placental 25(OH)D$_3$ metabolism (16, 18–21), but in a recent study, Hollis *et al.* (22) suggested that human placenta may not express the enzymatic pathways necessary to synthesize 24,25(OH)$_2$D$_3$. The present investigations used cochromatography with internal authentic standards to advantage to identify placental 24,25(OH)$_2$D$_3$ more precisely and to localize its production to cyto- and syncytiotrophoblast. We identified the compound unequivocally by its UV spectrum and by mass spectrometry of both the native metabolite and its periodate cleavage product. Therefore, the disparity between our results and those of Hollis *et al.* (22) is difficult to explain. It is possible that their method of tissue preparation, which involved slow freezing and thawing of placental homogenates in the absence of protease inhibitors, may have permitted significant degradation of placental enzymes.

Renal 24-hydroxylation appears to be catalyzed by a distinct cytochrome P450 enzyme encoded by a novel gene (43, 44). Ordinarily, 24-hydroxylase activity is not activated unless vitamin D target cells are stimulated by the administration of 1,25(OH)$_2$D$_3$ (27). This proved to be the case for placental fibroblasts, because the cells metabolized 25(OH)D$_3$ when they were preincubated with 1,25(OH)$_2$D$_3$ but not after culture in vitamin D-deficient media. In contrast, we have shown that a remarkable feature of trophoblast 24-hydroxylation is its high basal activity, even when culture conditions should have depleted the tissue of vitamin D metabolites. It is also unlikely that 1,25(OH)$_2$D$_3$ present at the time of tissue harvesting was retained during the 2- to 4-d culture interval, because placenta *in vitro* very rapidly and efficiently metabolizes 1,25(OH)$_2$D$_3$ via the $C_{24}$ and $C_{23}$ oxidation pathways (45).

In the intact, vitamin D-replete kidney, synthesis of 24,25(OH)$_2$D$_3$ is the major pathway for 25(OH)D$_3$ metabolism (46). *In vitro* administration of exogenous 1,25(OH)$_2$D$_3$ in a dose-dependent fashion increases recovery of 24,25(OH)$_2$D$_3$ and decreases recovery of 1,25(OH)$_2$D$_3$ in kidney (25, 26) and in extrarenal sites of 1,25(OH)$_2$D$_3$ synthesis (47, 48). In contrast to kidney, placenta apparently uses 24-hydroxylation as the predominant 25(OH)D$_3$ metabolic pathway regardless of the vitamin D status of the tissue.

Conceivably, this high basal 24-hydroxylase activity explains why we could not consistently demonstrate 1,25(OH)$_2$D$_3$ production when placenta was incubated with physiologic concentrations of 25(OH)D$_3$, despite the use of very sensitive and specific HPLC systems to analyze vitamin D$_3$ metabolites. These data are consistent with two previous reports that could not convincingly demonstrate 1,25(OH)$_2$D$_3$ production either by human placenta incubated with 6 nM 25(OH)D$_3$ (20) or by rat yolk sac (a human placental homolog) incubated with 10 nM 25(OH)D$_3$ (49). In fact, all analytical characterization of placental 25(OH)D$_3$-1-hydroxylation *in vitro* has derived from studies of placental homogenates incubated with concentrations of 25(OH)D$_3$ (40–150 μM) (16, 19, 21, 22), which are much greater than the apparent Km for the 24*R*- and 1α-hydroxylases [235 nM and 890 nM, respectively, for the rat renal mitochondrial

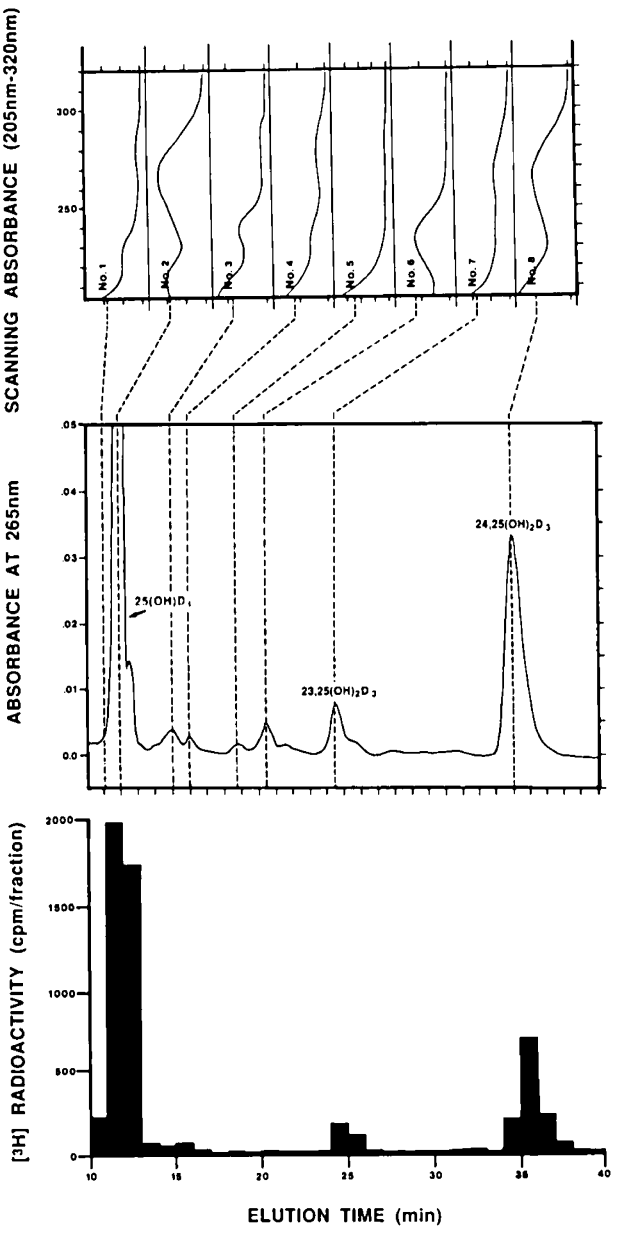


Fig. 3. HPLC analysis of the lipid extract of human trophoblast tissue. Placental explants were incubated for 20 h with 10 $\mu$M 25(OH)$D_3$ (including 4 $\mu$Ci [$^3$H]25(OH)$D_3$). *Middle panel*, one tenth of the lipid extract (tissue plus medium) was chromatographed on a Zorbax-SIL column eluted with a 2% isopropanol:hexane solvent system. UV absorbance was recorded at 265 nm. *Top panel*, scanning spectrophotometry (205–320 nm) of each peak detected on the $\lambda_{265}$ trace indicates that three peaks contain the signature vitamin $D_3$ triene chromophore, namely, nos. 2, 7, and 8, which eluted in the expected positions of 25(OH)$D_3$, 23,25(OH)$_2D_3$, and 24,25(OH)$_2D_3$, respectively. *Bottom panel*, the corresponding elution profile for the $^3$H radioactivity verifies that [$^3$H]25(OH)$D_3$ was metabolized to putative [$^3$H]23,25(OH)$_2D_3$ and [$^3$H]24,25(OH)$_2D_3$.

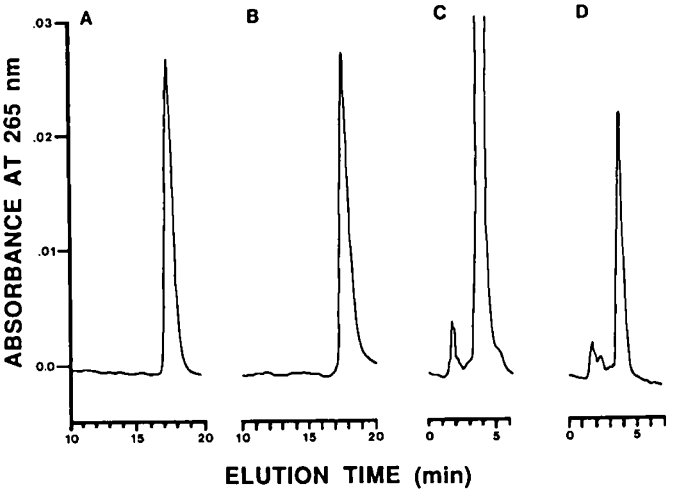


Fig. 4. HPLC identification of placental 24,25(OH)$_2D_3$ and its periodate cleavage product. Placental explants were incubated for 20 h with 10 $\mu$M 25(OH)$D_3$. The lipid extracts were purified on a Zorbax-SIL column using a 2% isopropanol:hexane, and the putative placental 24,25(OH)$_2D_3$ peak was then rechromatographed using a methylene chloride–based solvent system. The final purified placental metabolite eluted as a single peak (*A*) with a retention time identical to that of the 24,25(OH)$_2D_3$ standard (*B*). A portion of the putative placental 24,25(OH)$_2D_3$ peak was then subjected to periodate cleavage as described in Materials and Methods. Sensitivity to periodate was demonstrated by HPLC using a 2% isopropanol:hexane solvent. The periodate cleavage products of the purified placental 24,25(OH)$_2D_3$ (*C*) and of the 24,25(OH)$_2D_3$ standard (*D*) eluted as single peaks with identical retention times in this system.

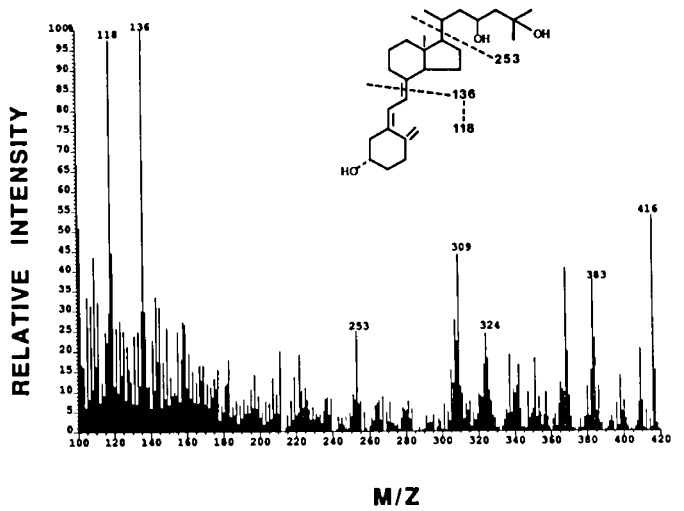


Fig. 5. Mass spectrum of the purified human placental 24,25(OH)$_2D_3$ periodate cleavage product, 25,26,27-trisnor(OH)$_2D_3$-24-al. Structure of the compound and fragmentation pattern are shown in the insert.

enzymes (50)]. Only in this fashion, *i.e.* when we administered 6 to 10 $\mu$M 25(OH)$D_3$ to cultured trophoblast or villous explants, could we consistently isolate a metabolite that possessed the chromatographic properties of 1,25(OH)$_2D_3$ in three different HPLC systems. Our results suggest that this substrate range, which exceeds plasma 25(OH)$D_3$ concentrations several-fold, saturates placental 24-hydroxylase and permits placental 1,25(OH)$_2D_3$ accumulation by inhibiting the further metabolism of 1,25(OH)$_2D_3$ by 24-hydroxylation. Because trophoblast 24-hydroxylase activity apparently is not saturated under physiologic conditions, we speculate that 1,25(OH)$_2D_3$ synthesized locally in placenta may be rapidly catabolized, principally via $C_{24}$ oxidation. Our preliminary observations indicate that this occurs when placenta is exposed to exogenous 1,25(OH)$_2D_3$ (45).

We speculate that another consequence of this high placental 24-hydroxylase activity might be the effective partition of the maternal and fetal circulations with respect to vitamin $D_3$ metabolites. Human placenta performs a similar function in perinatal corticosteroid metabolism. In that instance, high levels of fetal plasma cortisone have been explained by the activity of placental 11$\beta$-dehydrogenase, which converts maternal cortisol to fetal cortisone (51, 52). Similarly, placental 24-hydroxylase

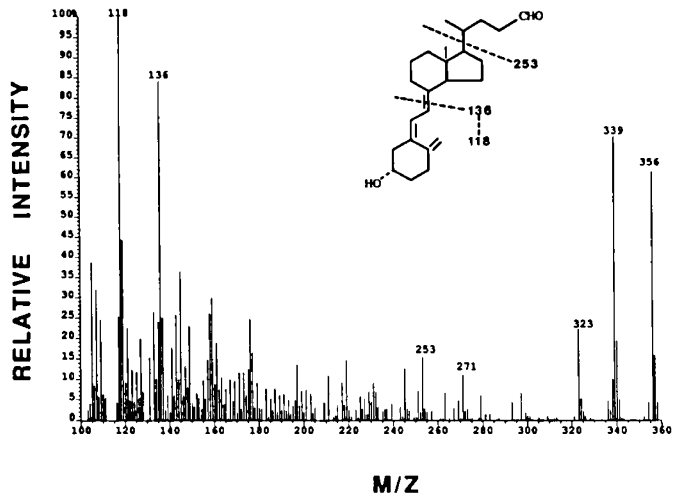


Fig. 6. Mass spectrum of purified putative human placental 23,25(OH)$_2$D$_3$. Structure of the compound and fragmentation pattern are shown in the insert. Fragments of m/z ≥ 250 are amplified 5-fold.

activity might contribute to the 24,25(OH)$_2$D$_3$ levels that have been observed in fetal (umbilical venous) plasma (29, 53–55) and amniotic fluid (56). Finally, this accumulation of placental 24,25(OH)$_2$D$_3$ may have important implications for fetoplacental function, because 24,25(OH)$_2$D$_3$ appears to be a unique vitamin D hormone with actions distinct from those of 1,25(OH)$_2$D$_3$. Specific 24,25(OH)$_2$D$_3$ receptors have been demonstrated in bone (57–59) and parathyroid (60). Developmentally regulated tissue responsiveness to 24,25(OH)$_2$D$_3$ is exhibited by bone cells (5, 10, 59), kidney (61), and nerve tissue (10), and 24,25(OH)$_2$D$_3$ may have distinct activities in (especially embryonic) bone formation and mineralization (4, 9, 12, 62) and intestinal calcium absorption (63).

We have also shown that 23-hydroxylation is a second, albeit less favored, pathway for placental 25(OH)D$_3$ metabolism. Placental 23,25(OH)$_2$D$_3$ production was unequivocally demonstrated by chromatography with authentic standard and by structural analysis (resistance to periodate cleavage and mass spectrometry). This report is the first direct demonstration of 23,25(OH)$_2$D$_3$ production by the placenta. In most mammalian systems studied to date, guinea pig kidney being a notable exception (64), the predominant side-chain dihydroxylated vitamin D$_3$ metabolite is 24,25(OH)$_2$D$_3$, not 23,25(OH)$_2$D$_3$, although some of each compound is made (65). Renal 23-hydroxylases are optimally expressed only after long-term treatment with high doses of vitamin D metabolites (41, 66, 67), and this also proved to be the case for placenta. The molar ratio of accumulated placental 23,25(OH)$_2$D$_3$ to 24,25(OH)$_2$D$_3$ also approximated that reported for renal systems. The fate and functions of 23,25(OH)$_2$D$_3$ and its main further metabolites, especially 25(OH)D$_3$-26,23-lactone and 1,25(OH)$_2$D$_3$-26,23-lactone, are under active investigation, as discussed in a recent review (68). Hence, the functions of placental 25(OH)D$_3$-23 hydroxylation remain to be defined.

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