

Vitamin D Supply to the Rat Fetus and Neonate

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Abstract

The prevention of neonatal rickets by oral supplementation with vitamin D₂ (ergocalciferol) has tended to obscure our ignorance of the natural mechanism by which young mammals receive an adequate supply of vitamin D. To investigate the possibility of specific intrauterine transfer and storage of vitamin D in fetal tissues, vitamin D-deficient female rats were given depot injections of ³H- or ¹⁴C-labeled vitamin D₃ (cholecalciferol) before mating and the ³H-labeled animals were killed at stages during the last third of gestation. Analysis of lipid extracts from whole fetuses revealed a linear increase in the concentration of 25-hydroxyvitamin D₃, 24,25-dihydroxyvitamin D₃, and D₃ itself between days 14 and 19 of gestation. During this period the elimination half-time of ³H-labeled molecules in maternal plasma fell from 27.1 to 4.4 d, suggesting that a specific mechanism was transferring vitamin D molecules into the fetuses. The vitamin was stored predominantly as 25-hydroxyvitamin D₃ and 24,25-dihydroxyvitamin D₃, with the highest concentrations in fetal muscle. Immediately after birth, pups from ³H- and ¹⁴C-labeled mothers were exchanged and later killed after 1–3 wk of suckling. Analysis of total lipid extracts for ³H and ¹⁴C content determined the relative contributions of vitamin D supplied before birth via the placenta and after birth in the maternal milk. The vitamin D content of the rat milk was relatively high, between 1.0 and 3.5 µg/liter. Nevertheless, the supply of vitamin D in utero, rather than from milk, was the main determinant of vitamin D status in early neonatal life. This is the first indication in a mammal of a specific transfer mechanism that allows the fetus to accumulate vitamin D from the mother during the last third of gestation.

Introduction

The natural source of vitamin D for young mammals in the early postnatal period is not readily apparent (1). The vitamin D content of mammalian milk is low (< 1 µg/liter) (2–4) and in many species the cutaneous synthesis of vitamin D is limited because the newly born are reared away from direct sunlight. Thus the input of vitamin D soon after birth, either from

ultraviolet irradiation of skin or from the diet, is restricted. Yet the requirement of vitamin D for the human baby, for example, is said to be as high as 10 µg/d (5, 6). It would appear that this amount cannot be obtained without artificial supplementation.

The plasma concentration of 25-hydroxyvitamin D (25(OH)D)¹ in mammalian offspring at birth is closely related to the mother's (7–9). When maternal vitamin D status is low, neonatal rickets (10) and hypocalcemia (11) can occur. The incidence of these signs of vitamin D deficiency shows a seasonal pattern which parallels the seasonal variation in maternal vitamin D status (11–13). Vitamin D and 25(OH)D cross the mammalian placenta readily during gestation (14), and because the 25-hydroxylase in the fetal liver may have low activity (15), fetal requirements are met directly from the maternal circulation.

In birds a special mechanism exists that enables maternal cholecalciferol to be specifically transferred and accumulated in egg yolk (16). This is then used during embryonic growth and for several days after the chick hatches. It is therefore possible that a similar mechanism exists in mammals whereby vitamin D could quantitatively accumulate in the fetus to meet postnatal needs. If this were so the prenatal supply of vitamin D would be the important determinant of neonatal vitamin D status in mammals rather than the postnatal supply from milk or solar irradiation of skin. The possibility of a special mechanism for quantitative transfer of maternal vitamin D to the fetus has been investigated in rats. In these experiments two different radiolabeled forms of vitamin D have been used to compare the quantity of vitamin D supplied to the rat fetus in utero with that which is subsequently delivered in the maternal milk during suckling.

Methods

Animal protocols. Female Norwegian hooded rats were housed in the absence of ultraviolet light and fed from weaning on a semisynthetic vitamin D-deficient diet (17). The female offspring of these animals were raised under the same conditions and used for breeding when they weighed ~ 215 g. The plasma of these second-generation, vitamin D-deficient rats contained no detectable 25(OH)D on HPLC (18) and the concentration of 1,25-dihydroxyvitamin D measured by RIA (18) was only 30–40 pg/ml (normal range 78±6 pg/ml).

12 of these vitamin D-deficient rats were injected with either ¹⁴C- or ³H-labeled vitamin D so that the deficiency was corrected and the absolute amount of vitamin D and its metabolites found in the plasma and tissues could be readily calculated from the concentration of radioactivity. The ¹⁴C-labeled vitamin D (¹⁴C-D₃) ([4-¹⁴C]cholecalciferol, 55.0 mCi/mmol, Amersham Corp., Arlington Heights, IL) was diluted with unlabeled cholecalciferol to a specific activity of 26 µCi/mmol (150 dpm/ng). The ³H-labeled vitamin D (³H-D₃) ([1α,2α(n)-³H]cholecalciferol, 25 Ci/mmol, Amersham Corp.) was diluted with unlabeled

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1. Abbreviations used in this paper: ¹⁴C-D₃, ¹⁴C-labeled vitamin D; ³H-D₃, ³H-labeled vitamin D; 24,25(OH)₂D, 24,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D.

beled cholecalciferol to a specific activity of 93.6 $\mu\text{Ci}/\text{mmol}$ (540 dpm/ng).

Three rats were each repleted with 20 μg ^{14}C -D₃ given as two subcutaneous injections of 5 μg ^{14}C -D₃ in 100 μl propylene glycol 48 h apart with an intramuscular depot injection of 10 μg ^{14}C -D₃ in 100 μl arachis oil into the right hind limb at the time of the second dose. Nine rats were each repleted with 20 μg ^3H -D₃ in the same manner.

1 d after the last injection the animals were bled by direct cardiac puncture (200- μl samples into heparinized tubes) under methoxy-fluorane anesthesia (PENTHRANE; Abbott Laboratories, Irving, TX) and vitamin D status was assessed by measuring the radioactivity in 50 μl plasma in a "Tri-Carb" liquid scintillation spectrometer (model 2650; Packard Instrument Co., Inc., Downers Grove, IL) using Triton X-100/toluene scintillator solution.

2 d after the last injection the ^{14}C -dosed rats and seven of the ^3H -dosed rats were paired for mating with normal male rats previously fed a standard rodent laboratory diet. Mating was dated from the appearance of vaginal plugs in trays under the animals' wire cages. Two of the ^3H -dosed rats remained unmated as controls and were bled at five intervals over the following 2 mo to establish the long-term elimination half-time of ^3H -D₃ metabolites in the plasma.

One ^3H -dosed rat was killed on each of days 14, 17, 19, and 21 of gestation. Each rat was anesthetized with diethyl ether and exsanguinated by cardiac puncture. Vitamin D status was assessed by measuring the radioactivity in 50 μl plasma and the remaining plasma was stored at -20°C for subsequent chromatographic analysis of ^3H -D₃ metabolites. Individual fetuses were dissected from the uterus, weighed, and stored at -20°C . The maternal right hind leg containing the residual intramuscular depot injection of ^3H -D₃ in arachis oil was also stored for later analysis.

The remaining three ^3H -dosed rats and the three ^{14}C -dosed rats each gave birth to between 11 and 15 pups on day 22 of gestation. The litters were culled to eight pups each within 2 to 4 h after birth and those from the ^3H -labeled mothers were exchanged with those from the ^{14}C -labeled mothers before significant suckling had taken place. This exchange enabled the relative quantity of vitamin D and its metabolites obtained in utero to be compared with that obtained postnatally from milk, by the simple analysis of ^3H and ^{14}C in the tissues of pups at intervals after birth. The pups were killed with an excess of diethyl ether anesthetic and were dissected and their organs were weighed and stored at -20°C for analysis.

One ^3H -labeled mother, a corresponding ^{14}C -labeled mother, and all their fostered pups were killed at weekly intervals after birth and the tissues were stored for analysis. Milk was collected at weekly intervals by suction from the mammary glands after oxytocin stimulation as described for mice (19).

Laboratory methods. Plasma and milk samples were diluted with an equal volume of 0.9% NaCl in H₂O and lipid was extracted with 2 vol of isopropanol/toluene (25:75, vol/vol) (20). Individual fetuses and the organs of newborn pups were macerated using fine scissors in widemouthed tubes and the blades of the scissors were wiped with small pieces of absorbant tissue which were added to the mixture to avoid any loss. Whole rat pups and maternal hind limbs were individually macerated in a manually operated domestic meat mincer and the tissues were transferred to separating funnels. The mincer was also cleaned with absorbant tissue which was added to the minced preparation. Total lipid was extracted from all these samples with chloroform/methanol (1:2, vol/vol) (21).

Because of the large number of samples to be analyzed, a simple chromatographic analysis of radiolabeled vitamin D metabolites was done using Sep-Paks (Silica Products Co., Inc., Guinon, AR) (20). A proportion of each lipid extract was evaporated under a stream of nitrogen at 30°C , redissolved in 2 ml 0.1% isopropanol in hexane, and applied to a Sep-Pak (Silica Products Co., Inc.) cartridge washed 2 h previously with 5 ml hexane. Neutral lipids, vitamin D₃, 25(OH)D₃, 24,25-dihydroxyvitamin D₃ (24,25(OH)₂D₃), and more polar vitamin D metabolites, which included 1,25-dihydroxyvitamin D₃ were eluted successively with specific solvent mixtures (20). The eluted solvents

were collected into plastic vials, evaporated overnight under a stream of air, and the radioactivity was measured by liquid scintillation spectrometry. The percentage distribution of vitamin D metabolites in the lipid extract was calculated from the distribution of radioactivity in the eluted fractions. A similar procedure for quantifying vitamin D metabolites has been described by Reinhardt et al. (22).

The repeatability and accuracy of this method was established using lipid extracts containing added standard radiolabeled vitamin D metabolites. Under these conditions 98.8% of vitamin D₃ present eluted with the second solvent (1% isopropanol in hexane), 91.7% of 25(OH)D₃ present eluted with the third solvent (3% isopropanol in hexane), and 60.2% of 24,25(OH)₂D₃ present eluted with the fourth solvent (3.125% ethanol in dichloromethane).

A proportion of the lipid extracts from fetuses and pups of the same litter were pooled and chromatographed on Sephadex LH20 (60 \times 1 cm) with chloroform/hexane (65:35, vol/vol) and there was found to be no significant difference between the mean distribution of radiolabeled vitamin D₃, 25(OH)D₃, and 24,25(OH)₂D₃ established by this technique, and that obtained by the Sep-Pak method. The identity of specific metabolites eluting from either the Silica Sep-Paks or Sephadex LH20 was further confirmed by HPLC (18), and in the case of 24,25(OH)₂D₃, by periodate cleavage (23).

Results

The pattern of fetal growth between days 14 and 22 of gestation was found to closely parallel the established standard growth curve (24), which confirmed that fetal gestational age had been accurately dated. The total content of vitamin D molecules in individual fetuses increased rapidly during the last third of gestation, going from 0.36 ± 0.02 pmol/fetus (mean \pm SEM) at day 14 to 62.2 ± 3.3 pmol/pup at birth (Fig. 1).

The major metabolite in the fetuses at all times was 25(OH)D₃, with lesser quantities of 24,25(OH)₂D₃ and a small amount of unchanged vitamin D₃ itself (Fig. 2 and Table I). There was a linear increase in the total fetal concentrations of all three metabolites from days 14 to 19 of gestation (Fig. 2). During this same period the elimination half-time of ^3H in the maternal plasma was reduced to 4.4 d, compared with 27.1 d in the plasma of unmated control animals (Fig. 3), suggesting that a specific mechanism was quantitatively transferring vitamin D molecules from the maternal plasma into the fetal tissues. Analysis of the hind legs of mothers killed on days 14–22 of gestation revealed that between 36.7 and 40.1% of the original intramuscular depot injection of radiolabeled D₃ re-

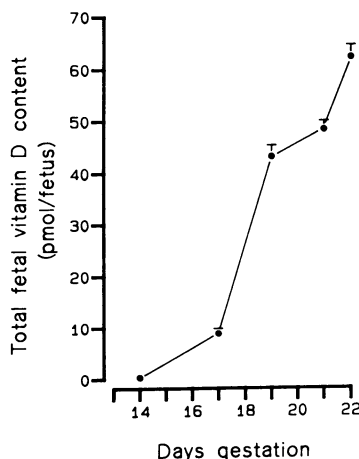


Figure 1. Increase in total content of vitamin D-derived molecules (mean \pm SEM, $n = 10$ –26) in whole rat fetuses during the last third of the gestation period.

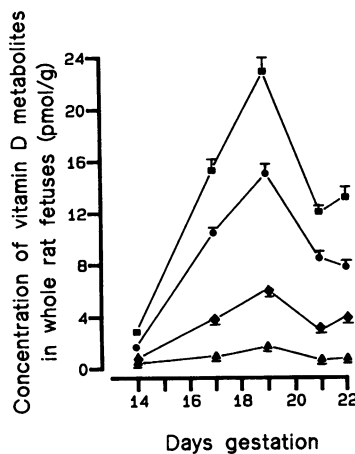


Figure 2. Change in concentrations (mean±SEM, $n = 10-26$) of total vitamin D-derived molecules (■), 25(OH)D₃ (●), 24,25(OH)₂D₃ (◆), and D₃ (▲) in whole rat fetuses during the last third of the gestation period.

maintained. Maternal vitamin D status was very low by the end of gestation and remained so over the following 3 wk (Fig. 3).

The distribution of the major vitamin D metabolites in the pup tissues at birth was similar to the maternal plasma except for a lower proportion of unchanged vitamin D itself (Table I). The highest tissue concentration of vitamin D and its metabolites was found in the skeletal muscle of the pup hind limbs and somewhat lower concentrations were found in the skin, stomach, carcass, intestines, and liver (Table I).

The vitamin D content of the maternal milk was relatively high initially ($\sim 3 \mu\text{g/liter}$), perhaps reflecting its high lipid content ($19.2 \pm 1.6\%$, mean±SEM), but fell progressively during the first 3 wk of lactation (Fig. 4). The vitamin was present mainly as unchanged D₃ during the first week of lactation but this also declined rapidly so that after 10 d the predominant metabolite was 25(OH)D₃ (Table II). We suspect that the presence of milk explained the higher proportion of D₃ in the stomach and intestines of the newborn pups (Table I).

The total vitamin D content of the pup tissues gradually declined during the first three weeks after birth (Table III) and plasma levels of 25(OH)D₃ eventually fell below 3 ng/ml (Fig. 5). Comparison of the ratio of ³H- to ¹⁴C-labeled vitamin D metabolites showed that the vitamin received in utero was gradually being replaced by that from the maternal milk (Fig. 6). Nevertheless vitamin D molecules obtained in utero, rather

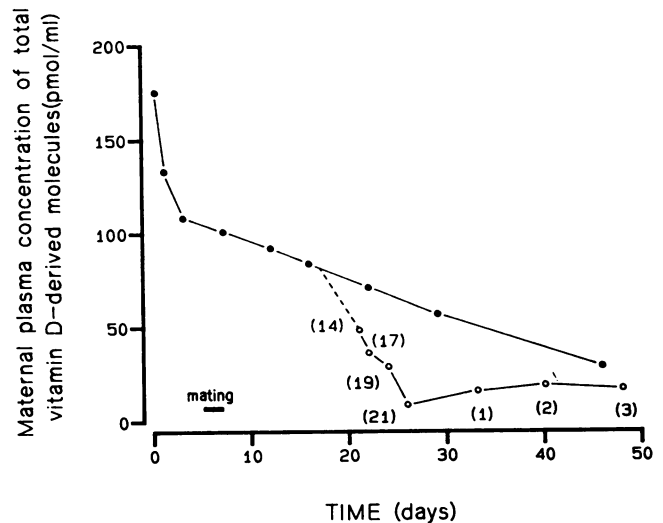


Figure 3. Mean plasma concentrations of total vitamin D-derived molecules in two unmated female control rats (●), compared with values in individual pregnant rats (○) killed on days 14, 17, 19, and 21 of gestation, and 1, 2, and 3 wk after giving birth. The elimination half-time ($t_{1/2}$) for the control curve was 27.1 d, while for the pregnant rats between days 14 and 21 of gestation $t_{1/2}$ was 4.4 d.

than from milk, were the main determinants of vitamin D status during early neonatal life.

Discussion

The classical form of vitamin D-deficiency rickets is described as a disease of children from 6 to 24 mo of age (25). Although rickets can occur in utero (26) and in the neonatal period (10) if maternal vitamin D status is particularly low, the relatively rare occurrence of vitamin D deficiency in the first few months after birth has not been explained (25). One possible reason for this apparent protection against rickets in the early neonatal period could be that a store of vitamin D is laid down in fetal tissues in utero. Such a mechanism for maintaining neonatal vitamin D status is revealed by this study in the rat. Vitamin D and 25(OH)D can cross the placenta to meet the vitamin D requirements for fetal growth (14). However the results in

Table I. Vitamin D Metabolites in Pup Tissues and Maternal Plasma at Birth

Pup tissue	Total concentration of vitamin D-derived molecules <i>pmol/g</i>	% Distribution of vitamin D metabolites (mean±SEM, $n = 10$)			
		D ₃	25(OH)D ₃	24,25(OH) ₂ D ₃	More polar metabolites
	(Muscle: 16.5 ± 3.2)				
Hind limbs	14.7 ± 0.7	5.4 ± 1.1	67.4 ± 2.7	18.2 ± 2.2	9.1 ± 1.1
	(Skin: 14.3 ± 2.3)				
Stomach	13.1 ± 2.7	50.1 ± 3.5	20.6 ± 2.1	15.0 ± 1.5	14.4 ± 2.5
Carcass	12.4 ± 0.7	4.8 ± 0.4	71.4 ± 3.1	17.8 ± 3.4	6.1 ± 0.6
Intestine	11.3 ± 1.6	14.0 ± 0.3	41.4 ± 0.9	24.2 ± 0.5	20.5 ± 0.7
Liver	7.5 ± 0.9	7.9 ± 1.3	30.2 ± 2.7	22.7 ± 3.6	39.3 ± 6.4
Plasma (<i>pmol/liter</i>)	48.7 ± 2.5	—	—	—	—
Maternal plasma*	8.7	18.8	54.1	20.4	3.1

* Means of two samples.

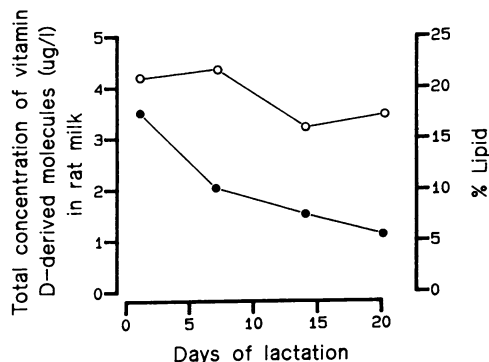


Figure 4. Change in total concentration of vitamin D-derived molecules (●) and lipid content (○) of rat milk during the first 3 wk of lactation. Values are means of two samples.

these experiments show that the vitamin D content of whole rat pups increases markedly during the last third of the gestation period, particularly between days 17 and 19 (Fig. 1), and this increase occurs in advance of the period of most rapid weight gain (days 19–21) so that there is a steady rise in concentration (Fig. 2). Furthermore, the increase in the total fetal content of vitamin D-derived molecules between days 14 and 21 was sufficient to quantitatively account for the striking fall in the vitamin D concentration in maternal plasma over the same period (Fig. 3). This suggests a specific enhancement of placental transport of vitamin D during the last third of the gestation period.

It is notable that the fetal concentrations of all three major forms of vitamin D (D, 25(OH)D and 24,25(OH)₂D) increased in the same proportion (Fig. 2), which suggests that the mechanism of enhanced placental transfer is not specific for any one molecular species. While it is conceivable that only the transport of D itself was enhanced and that the rise in 25(OH)D and 24,25(OH)₂D levels was due to subsequent fetal metabolism, this is unlikely because 25(OH)D is known to cross the placenta (14) and the hepatic 25-hydroxylase in the fetus has relatively low activity (15). In contrast, in birds, vitamin D itself (cholecalciferol) is specifically accumulated in the egg, by a mechanism involving a cholecalciferol-specific binding protein (DBP) in the plasma of hens (16). Therefore a similar DBP-mediated transfer may be implicated in the enhanced transport of vitamin D and its metabolites into the rat fetus during the last third of the gestation period.

The vitamin was predominantly stored as 25(OH)D and its highest concentration was found in fetal muscle while the lowest was in the liver (Table I). There is very little subcutaneous

Table II. Vitamin D Metabolites in Rat Milk during the First 3 Wk of Lactation

Days of lactation	% Distribution of vitamin D metabolites*			
	D ₃	25(OH)D ₃	24,25(OH) ₂ D ₃	More polar metabolites
1	75.5	14.6	5.4	4.8
7	54.9	29.7	12.4	2.9
14	32.9	44.9	8.6	13.7
20	36.3	45.5	8.7	9.5

* Mean values on milk from two rats.

fat in the rat fetus, and muscle tissue forms the greatest proportion of total fetal mass. The capacity for accumulation and storage of significant quantities of vitamin D molecules in the fetus by this mechanism is therefore large. The liver is not a major storage site for vitamin D (1) and the higher proportion of D compared with 25(OH)D in the stomach and intestines of the newborn pup suggests that this reflects maternal milk in the gastrointestinal contents rather than specific accumulation (Tables I and II). The cellular mechanism of storage is not known but the rapid disappearance of all forms of vitamin D from tissues during the first 3 wk after birth (Fig. 6) suggests that this is readily available to meet early neonatal requirements.

The total vitamin D concentration in rat milk (Fig. 4) during the first 3 wk of lactation (1.0–3.5 μg/liter) was similar to that reported in human and bovine milk, which varies between 0.1 and 1.0 μg/liter depending on the extent of maternal vitamin D supplementation (3). The rather higher total quantities of vitamin D that we found in rat milk during the first 14 d of lactation may reflect the higher proportion of fat in the latter (19–20%) (27) compared with human and bovine milk (4–5%), although the same change in vitamin D content with time is also seen in cow's milk (28). In the very early stages of lactation the vitamin was mainly present in the rat milk (or colostrum) as D itself, but within 14 d the D content had declined (Table II) so that the predominant metabolite was 25(OH)D. The vitamin D content of milk can be significantly increased by maternal supplementation (3), but the pattern of vitamin D metabolites in milk suggests that these enter by simple diffusion from plasma.

From the relative proportions of ³H and ¹⁴C in the rat pups (Fig. 6) it is clear that the major source of vitamin D molecules during the first 10 d after birth is that which is supplied and stored while in utero rather than that obtained from milk. This is apparent despite the fact that the rat pups were culled to

Table III. Total Vitamin D Metabolites in Whole Rat Pups during the First 3 Wk after Birth

Age	Total content of vitamin D-derived molecules	% Distribution of vitamin D metabolites (mean±SEM)			
		D ₃	25(OH)D ₃	24,25(OH) ₂ D ₃	More polar metabolites
wk	pmol/pup				
Birth (n = 26)	62.2±3.3	3.0±0.2	58.3±1.2	29.0±1.2	10.4±0.6
1 (n = 14)	50.1±2.7	5.8±0.2	50.7±1.1	29.4±0.9	14.2±0.6
2 (n = 13)	44.8±3.5	5.9±0.4	45.0±1.7	23.7±1.2	25.4±2.3
3 (n = 8)	38.5±2.0	7.3±0.6	57.2±4.1	17.7±1.1	17.8±1.9

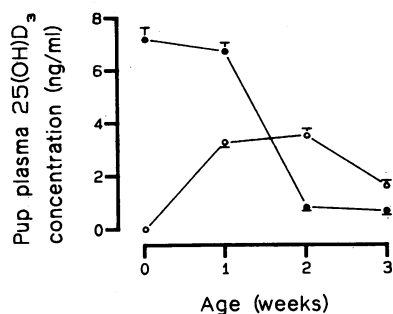


Figure 5. Plasma concentrations of 25(OH)D₃ derived from vitamin D received in utero (●) compared with that derived from milk (○) during the first 3 wk after birth.

eight per litter at birth and therefore the milk contribution to the vitamin D status of the remaining pups was exaggerated. Furthermore since we repleted the vitamin D-deficient rat mothers with parenteral vitamin D only once before mating, the maternal vitamin D status was rather low by the time of birth and remained so during the following weeks (Fig. 3). If a more effective dosing schedule had been used and maternal vitamin D status had been higher, it seems likely that fetal storage of vitamin D could have been increased and might have maintained the neonatal vitamin D status for a longer time. Optimal maternal vitamin D supply had clearly not been achieved in this experiment since the concentration of 25(OH)D in the pup plasma fell below 3 ng/ml within 3 wk of birth (Fig. 5), despite the fact that the maternal milk contained significantly more vitamin D than has been found in other mammalian species (2–4). The fall in pup plasma concentrations of 25(OH)D during the first few weeks after birth highlights both the inadequacy of vitamin D supply in milk and the possibility of persisting immaturity of the hepatic 25-hydroxylase in the early neonatal period since there is no change in the rate of utilization of 25(OH)D at this time (29).

In summary, we have shown for the first time in a mammal that there is a specific enhancement of the placental transport of vitamin D during the last third of the gestation period in the rat. The vitamin D metabolites (D₃, 25(OH)D₃, and 24,25(OH)₂D₃) are accumulated and stored in fetal tissues, mainly in muscle, where they are readily available for neonatal requirements. The neonate is therefore dependent on its mother's vitamin D status during gestation rather than on external sources for its vitamin D supply. If such a mechanism

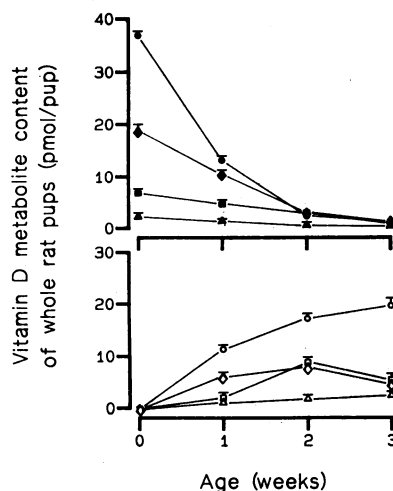


Figure 6. Change in whole rat pup content of vitamin D metabolites (mean ± SEM, $n = 8-26$) during the first 3 wk after birth. Vitamin D₃ (▲), 25(OH)D₃ (●), 24,25(OH)₂D₃ (◆), and more polar metabolites (■) received in utero (solid symbols) are gradually replaced by the metabolites derived from milk (open symbols).

were operating in other mammals, including man, it would explain the close association between neonatal rickets and maternal vitamin D deficiency seen particularly in the Asian population in the U.K. (10–13). It would also explain the relative efficacy of maternal supplementation with vitamin D during the last trimester of pregnancy in preventing rickets (30).

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References

- Fraser, D. R. 1983. The physiological economy of vitamin D. *Lancet*. i:969–972.
- Macy, I. G., and H. J. Kelly. 1961. In *Milk: The Mammary Gland and its Secretion*. S. K. Kon and A. T. Cowie, editors. Academic Press, Inc., New York, London. 265.
- Hollis, B. W., B. A. Roos, H. H. Draper, and P. W. Lambert. 1981. Vitamin D and its metabolites in human and bovine milk. *J. Nutr.* 111:1240–1248.
- Hidioglou, M. 1984. Fate of tritium-labelled vitamin D₃ and 25-hydroxyvitamin D₃ in rabbit does and their pups. *J. Dairy Sci.* 67:76–81.
- Department of Health and Social Security. 1969. Recommended intakes of nutrients for the United Kingdom. Reports on Public Health and Medical Subjects, HMSO London. No. 120.
- Recommended Dietary Allowances. 1974. National Academy of Sciences, Washington, D.C.
- Hillman, L. S., and J. G. Haddad. 1974. Human perinatal vitamin D metabolism. I. 25-Hydroxyvitamin D in maternal and cord blood. *J. Pediatr.* 84:742–749.
- Barlet, J. P., B. Argemi, M. J. Gavico, and J. Lefèvre. 1978. Plasma concentrations of 25-hydroxyvitamin D in pregnant and lactating ewes and foetal and newborn lambs. *J. Endocrinol.* 79:149–150.
- Goff, J. P., R. L. Horst, and E. T. Littlejohn. 1984. Effect of sow vitamin D status and parturition on the vitamin D status of neonatal piglets. *J. Nutr.* 114:163–169.
- Ford, J. A., D. C. Davidson, W. B. McIntosh, W. M. Fyfe, and M. G. Dunnigan. 1973. Neonatal rickets in the Asian immigrant population. *Br. Med. J.* 3:211–212.
- Watney, P. J. M., G. W. Chance, P. Scott, and J. M. Thompson. 1971. Maternal factors in neonatal hypocalcaemia: a study in three ethnic groups. *Br. Med. J.* 2:432–436.
- Roberts, S. A., M. D. Cohen, and J. O. Forfar. 1973. Antenatal factors associated with neonatal hypocalcaemic convulsions. *Lancet*. ii:809–811.
- Purvis, R. J., W. J. McK. Barrie, G. S. Mackay, E. M. Wilkinson, F. Cockburn, N. R. Belton, and J. O. Forfar. 1973. Enamel hypoplasia of the teeth associated with neonatal tetany: a manifestation of maternal vitamin-D deficiency. *Lancet*. ii:811–814.
- Haddad, J. G., V. Boisseau, and L. V. Avioli. 1971. Placental transfer of vitamin D₃ and 25-hydroxycholecalciferol in the rat. *J. Lab. Clin. Med.* 77:908–915.
- Hillman, L. S., and J. G. Haddad. 1975. Perinatal vitamin D metabolism. II. Serial 25-hydroxyvitamin D concentrations in sera of term and premature infants. *J. Pediatr.* 86:928–935.
- Fraser, D. R., and J. S. Emtage. 1976. Vitamin D in the avian egg. *Biochem. J.* 160:671–682.
- DeLuca, H. F., G. Guroff, H. Steenbock, S. Reiser, and M. R. Mannatt. 1961. Effect of various vitamin deficiencies on citric acid metabolism in the rat. *J. Nutr.* 75:175–180.
- Mawer, E. B., J. T. Hann, J. L. Berry, and M. Davies. 1985. Vitamin D metabolism in patients intoxicated with ergocalciferol. *Clin. Sci. (Lond.)*. 68:135–141.

19. Hoffmann, F. A., G. Sawatzki, H. Schmitt, and B. Kubanek. 1982. A milker for mice. *Lab. Anim. Sci.* 32:387-388.
20. Redhwi, A. A., D. C. Anderson, and G. N. Smith. 1982. A simple method for the isolation of vitamin D metabolites from plasma extracts. *Steroids*. 39:149-154.
21. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37:911-917.
22. Reinhardt, T. A., R. L. Horst, J. W. Orf, and B. W. Hollis. 1984. A microassay for 1,25-dihydroxyvitamin D not requiring high-performance liquid chromatography: application to clinical studies. *J. Clin. Endocrinol. & Metab.* 58:91-98.
23. Holick, M. F., H. K. Schnoes, H. F. DeLuca, R. W. Gray, I. T. Boyle, and T. Suda. 1972. Isolation and identification of 24,25-dihydroxycholecalciferol, a metabolite of vitamin D₃ made in the kidney. *Biochemistry*. 11:4251-4255.
24. Donaldson, H. H. 1924. *In The Rat. Data and Reference Tables*. 2nd ed. Wistar Institute, Philadelphia. 173-174.
25. Fraser, D. R. 1980. Vitamin D. *In Vitamins in Medicine*. B. M. Barker and D. A. Bender, editors. Heinemann, London. 42-146.
26. Russell, J. G. B., and L. F. Hill. 1974. True fetal rickets. *Br. J. Radiol.* 47:732-734.
27. Roberts, S. B., and W. A. Coward. 1985. Dietary supplementation increases milk output in the rat. *Br. J. Nutr.* 53:1-9.
28. Okano, T., K. Yokoshima, and T. Kobayashi. 1984. High-performance liquid chromatographic determination of vitamin D₃ in bovine colostrum, early and later milk. *J. Nutr. Sci. Vitaminol.* 30:431-439.
29. Mendelsohn, M., and J. G. Haddad. 1975. Postnatal fall and rise of circulating 25-hydroxyvitamin D in the rat. *J. Lab. Clin. Med.* 86:32-37.
30. Mallet, E., B. Gugi, P. Brunelle, A. Henocq, J. P. Basuyau, and H. Lemeur. 1985. Vitamin-D supplementation in pregnancy: a controlled trial of 2 methods. *Obstet. Gynecol.* 68:300-304.