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Vitamin D Metabolism in Breast-Fed Infants and their Mothers

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ABSTRACT. The aim of this longitudinal study was to examine vitamin D metabolism in exclusively breast-fed infants. The four common vitamin D metabolites—25-hydroxyvitamin D (25OHD), 1,25-dihydroxyvitamin D [1,25(OH)$_2$D], 24,25-dihydroxyvitamin D [24,25(OH)$_2$D], and 25,26-dihydroxyvitamin D [25,26(OH)$_2$D]—as well as vitamin D binding protein (DBP) were determined simultaneously in mothers and their children from delivery to several months of age. Maternal blood samples, drawn approximately 6 wk before the expected date of delivery, were also analyzed. At delivery, total vitamin D metabolites in maternal and fetal plasma were closely correlated, maternal levels being higher. Unbound (free) vitamin D metabolite concentrations were higher in fetal than in maternal plasma, with the exception of free 1,25(OH)$_2$D levels, which were equal. This suggests a rapid placental transfer of 1,25(OH)$_2$D. 24,25(OH)$_2$D and 25,26(OH)$_2$D levels both in mothers and children were closely correlated with the precursor sterol 25OHD. For 1,25(OH)$_2$D, no correlation could be demonstrated with any of the other vitamin D metabolites. DBP concentrations in maternal plasma at the time of delivery were about twice the mean adult reference value. In cord blood, DBP levels were in the lower part of the adult reference range. Maternal total 1,25(OH)$_2$D levels, which were twice the reference mean during pregnancy, fell sharply after delivery but free 1,25(OH)$_2$D levels much less. Analogous to the biochemical changes in the mother, the infants' DBP levels fell after birth, as a result of the sudden disappearance of the estrogen stimulus. At the same time, the mineral supply via the placenta was cut off. These two factors are probably responsible for a stimulus to 1,25(OH)$_2$D synthesis (and/or inhibition of 1,25(OH)$_2$D degradation), resulting in a sharp increase of 1,25(OH)$_2$D and an even stronger increase of free 1,25(OH)$_2$D. Concentrations of 25OHD and 1,25(OH)$_2$D in breast milk were low. Such water-soluble metabolites as 25OHD-glucuronides were not detected. Judged by plasma 25OHD levels, the vitamin D stores of most children born to mothers with normal vitamin D status are depleted approximately 8 wk after delivery. Therefore, supplementation with an appropriate dose of vitamin D shortly after birth seems advisable, especially in winter. (*Pediatr Res* 25: 623–628, 1989)

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Abbreviations

25OHD, 25-hydroxycholecalciferol = 25-hydroxyvitamin D
24,25(OH)$_2$D, 24,25-dihydroxyvitamin D
25,26(OH)$_2$D, 25,26-dihydroxyvitamin D
1,25(OH)$_2$D, 1,25-dihydroxyvitamin D
DBP, vitamin D binding protein

Calcium metabolism in the pregnant woman and the fetus differs extensively from the usual adult pattern. Calcification of the fetal skeleton begins at the 8th wk. At birth, the neonate has accumulated about 30 g of calcium and 17 g of phosphorus (1). The maternal skeleton is protected against excessive bone resorption by high calcitonin levels (2). During pregnancy, the fetus builds up vitamin D stores. This is only possible if vitamin D and/or its metabolites are able to cross the placenta. As newborn infants are usually not exposed to direct sunlight, they have to rely on these stores.

The aims of this study were *1*) to obtain information about placental transfer of vitamin D metabolites by measuring them in maternal and cord plasma at birth; *2*) to estimate vitamin D stores in the newborn (indirectly) by longitudinal measurements of the four common vitamin D metabolites in plasma of exclusively breastfed babies; *3*) to investigate, by milk analyses, if human milk supplements these stores in exclusively breastfed infants.

MATERIALS AND METHODS

Subjects. The subjects were 39 pregnant, healthy Caucasian women in the 3rd trimester of pregnancy, who gave their informed consent to participate in this study. All children were born at term, most of them early in October, and were exclusively breastfed. Venous blood samples were drawn simultaneously from mother and infant at birth and after 1, 2, 3, 4, 8, 13, and 21 wk. The number of mother-child pairs was as follows: At birth, 39; 1st wk, 26; 2nd wk 21; 3rd wk 12; 4th wk, 9; 8th wk, 9; 13th wk, 5; 21st wk, 5. In addition, maternal blood samples were drawn approximately 6 wk before the expected date of delivery.

The four common vitamin D metabolites (see Table 1) and DBP were determined in all samples. After the 1st wk of life, matching breast milk samples were also obtained and kept at −20°C until assayed. There was no clinical evidence of osteo-

Table 1. *Plasma values on day of birth*

Compound	Unit	Fetomaternal difference*		p
		Maternal	Cord	
25OHD	nmol/liter	84 ± 6 (39)	55 ± 4 (38)	<0.001
$24,25(OH)_2D$	nmol/liter	2.7 ± 0.3 (39)	1.7 ± 0.2 (38)	<0.01
$25,26(OH)_2D$	nmol/liter	1.3 ± 0.1 (39)	0.7 ± 0.05 (38)	<0.001
$1,25(OH)_2D$	pmol/liter	155 ± 10 (39)	69 ± 7 (38)	<0.001
DBP	mg/liter	620 ± 13 (32)	284 ± 11 (31)	<0.001
25OHD free	pmol/liter	1.8 ± 0.15 (31)	2.7 ± 0.2 (31)	<0.001
$24,25(OH)_2D$ index	$mol/mol \cdot 10^4$	1.8 ± 0.2 (31)	2.4 ± 0.3 (31)	<0.05
$25,26(OH)_2D$ index	$mol/mol \cdot 10^4$	0.82 ± 0.07 (31)	1.0 ± 0.08 (31)	NS
$1,25(OH)_2D$ index	$mol/mol \cdot 10^5$	1.1 ± 0.07 (31)	1.1 ± 0.11 (31)	NS

* Values are mean ± SEM (*n*).

malacia or rickets in any of the mothers or their children, although after the 1st mo several children had elevated alkaline phosphatase levels and/or decreased 25OHD concentrations. All children showed normal growth.

Materials. Sephadex LH-20 was obtained from Pharmacia Fine Chemicals (Woerden, The Netherlands). 25OHD was a gift from Philips Duphar (Weesp, The Netherlands). $1,2(OH)_2D$, $24,25(OH)_2D$ and $25,26(OH)_2D$ were gifts from Hoffmann-La Roche (Mÿdrecht, The Netherlands). ^3H-labeled vitamin D metabolites were purchased from Amersham Corp. (Houten, The Netherlands). HPLC was performed using μ-Porasil and μ-Bondapak C18 columns in a Millipore-Waters system (Etten-Leur, The Netherlands).

Methods. Vitamin D metabolites in plasma. Plasma samples of 2 mL were extracted twice with 5 mL diethyl ether and once with 5 mL dichloromethane. The combined extracts were dried under nitrogen and purified on a 15- × 0.8-cm Sephadex LH-20 column eluted with hexane: chloroform:methanol (9:1:1; vol/vol). The fraction containing 25OHD was further purified by HPLC on a μ-Porasil column (isopropanol:hexane = 2:98; vol/vol). Purification of 25OHD was achieved by reversed phase HPLC, using a μ-Bondapak C18 column eluted with water:methanol (15:85; vol/vol).

Quantification was done with a UV-detector (254 nm). This method has intra- and interassay variations of 6 and 9%, respectively. The fraction containing the dihydroxylated vitamin D metabolites was purified on a silica μ-Porasil column eluted with isopropanol:hexane (7:93). Quantification of $24,25(OH)_2D$ and $25,26(OH)_2D$ was performed by a ligand binding assay (3) using human plasma as a source of binding protein. After 24 h of incubation, phase separation was carried out with dextran-coated charcoal. Quantification of $1,25(OH)_2D$ was done with a radioreceptor assay using the duodenal receptor from rachitic chickens (4).

For $24,25(OH)_2D$ and $25,26(OH)_2D$ the sensitivity of the assay was 12 pg/tube; for $1,25(OH)_2D$ 2 pg/tube. The intra- and interassay coefficients of variation were 10 and 18% for $24,25(OH)_2D$; 9 and 15% for $25,26(OH)_2D$; 11 and 15% for $1,25(OH)_2D$. All samples were corrected for recovery by means of tracers, added before extraction.

DBP and free vitamin D metabolites. Calculation of free vitamin D metabolite levels requires a total vitamin D (metabolite) assay and a DBP assay. The latter assay was performed according to Bouillon *et al.* (5). Measured and calculated free levels agree well if DBP values are not extremely low (6). For the calculation of free 25OHD, we used the numerical value of the k_d calculated by Bouillon *et al.* (7). If the exact k_d value was not known, the k_d was taken as unity, and free vitamin D metabolite concentrations were then expressed as the so-called free index, defined as the molar ratio of vitamin D metabolite and DBP.

Vitamin D metabolites in human breast milk. Human breast milk (10–15 mL) was brought to pH 8 with 0.1-N KOH and extracted twice with 30 mL diethyl ether and once with 30 mL dichloromethane. After every extraction, phases were allowed to separate at −20°C. The organic phases were pooled and dried under nitrogen. Sephadex LH-20 chromatography was performed as described above. For further (HPLC) purification of the 25OHD fraction, a μ-Porasil column was used, eluted with isopropanol:hexane (99:1; vol/vol). After every four samples, the column was stripped and re-equilibrated. Quantification of 25OHD was performed by a ligand binding assay as described for $24,25(OH)_2D$ and $25,26(OH)_2D$ measurement in plasma. The assay had a sensitivity of 12 pg/tube. The intra- and interassay coefficients of variation were 16% and 22%.

Vitamin D-25-glucosiduronate assay. Breast milk (20 mL) was brought to pH 5 with a concentrated acetate buffer. The final concentration was 0.1-M sodium acetate. Glucuronidase (5000 U) was added (Glucurase; Sigma Chemical Co., St. Louis, MO), and the samples were incubated at 37°C overnight, after which they were processed as described for human breast milk. In human breast milk spiked with vitamin D-25-glucosiduronate synthesized according to Nagubandi *et al.* (8), the recovery was 48% (measured as 25OHD).

STATISTICS

Student's *t* test and linear regression analysis were used as appropriate. The calculation of reference ranges for the vitamin D metabolites was performed as follows. Blood samples were drawn from 36 volunteers at random moments throughout the year. This relatively small set of "normal values" was used for the calculation of percentiles using a bootstrap technique described below (9).

From the original set of "normal values," entries were drawn at random to construct a new set of "normal values." At every random "draw," each concentration has an equal chance of being selected. After 99 "draws," the new set is complete. Some of the original values will occur several times; others will have escaped the drawing process. The new set allows the definition of the 90th, 80th, etc. percentile. This process was repeated 10^4 times, resulting in 10^4 values for each percentile. The resulting mean of each percentile was considered to be an adequate approximation of the "real" percentile. It was found that the final distributions very closely resembled log-normal distributions.

RESULTS

Plasma. The results obtained for total and free vitamin D metabolites and DBP are shown in Figures 1 and 2. No significant differences (Student's *t* test) were observed for any of the biochemical parameters on the d of birth between the mother-infant pairs who withdrew before the 21st wk and the five pairs who stayed in this study until its conclusion.

Table 1 presents a compilation of the data obtained from the analysis of maternal and cord plasma. On the d of birth, maternal plasma levels for 25OHD, $24,25(OH)_2D$, $25,26(OH)_2D$, $1,25(OH)_2D$, and DBP exceeded cord levels. Free 25OHD and

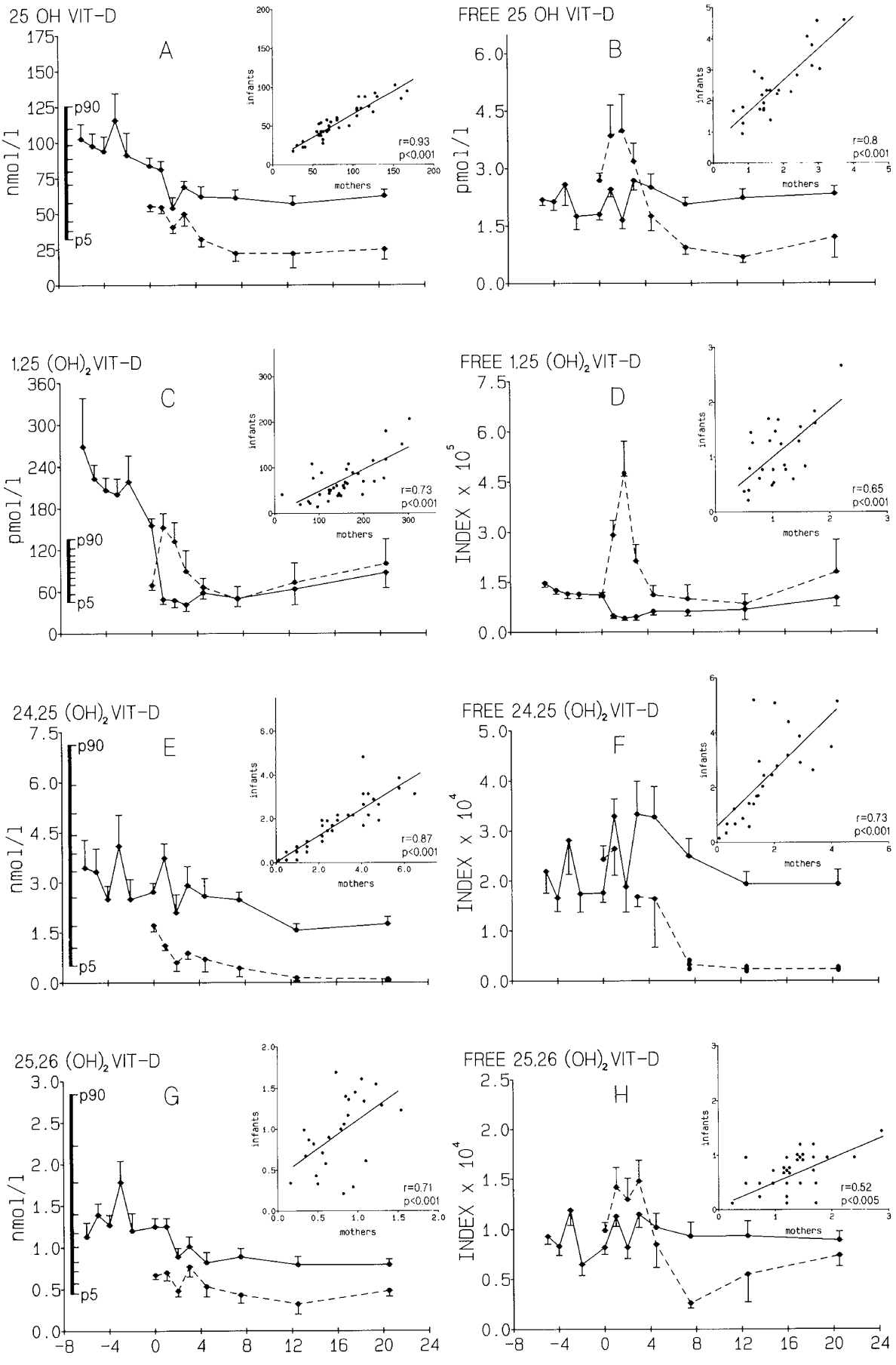

Fig. 1. Total and free vitamin D metabolite concentrations, mean ± SEM, in plasma of mothers (•——•) and children (•---•) *vs.* time in wk (0 = d of delivery). Insets represent the correlation between mothers and infants, at d of birth only. Vertical bars: reference ranges expressed as percentiles, p5, p10, p20, p30, p40, p50, p60, p70, p80, and p90 are indicated.

the 24,25(OH)$_2$D index were higher in cord than in maternal plasma. The 25,26(OH)$_2$D and 1,25(OH)$_2$D indices were not significantly different in maternal and infant plasma.

On the d of birth, a positive fetomaternal correlation existed for both total and free vitamin D metabolites (insets in Fig. 1). In the case of the 1,25(OH)$_2$D index, the slope of the regression line did not differ significantly from 1, indicating an almost perfect 1:1 relationship. DBP levels in maternal and cord plasma were not significantly correlated. In the mother during pregnancy, 25OHD, 24,25(OH)$_2$D and 25,26(OH)$_2$D were all significantly ($p < 0.001$) intercorrelated. On the day of birth, 25OHD was positively correlated with 24,25(OH)$_2$D and with 25,26(OH)$_2$D ($p < 0.001$) in both mothers and children. 24,25(OH)$_2$D and 25,26(OH)$_2$D were correlated at the $p < 0.01$ level (also in both mothers and in children).

At 1 wk postpartum, 25OHD and 24,25(OH)$_2$D were still correlated at the $p < 0.001$ level (mothers and children), but the significance level of the correlation between 25OHD and 25,26(OH)$_2$D in the mothers had dropped to $p < 0.01$. In the children, the correlation disappeared. The correlation between 24,25(OH)$_2$D and 25,26(OH)$_2$D had disappeared in the children, and in the mother not more than the $p < 0.05$ level was reached.

After the 3rd wk, no correlation between 25OHD and 24,25(OH)$_2$D could be demonstrated. As early as 1 wk after birth, no correlation could be demonstrated between 25,26(OH)$_2$D levels and its precursor 25OHD. For 1,25(OH)$_2$D, no correlation could be demonstrated with any of the other vitamin D metabolites, neither before delivery (mothers), or thereafter (mothers and children).

Breast milk. Levels of (unconjugated) 25OHD in breast milk are given in Figure 3. In milk, a mean value of 325 pmol/mL was found, 0.4% of the maternal plasma value. Milk and maternal plasma values were positively correlated ($p < 0.05$ or better) up to 5 wk after delivery. Figure 4 demonstrates the good correlation between maternal plasma and milk values of 25OHD in the 1st wk after birth. Only trace amounts of unconjugated

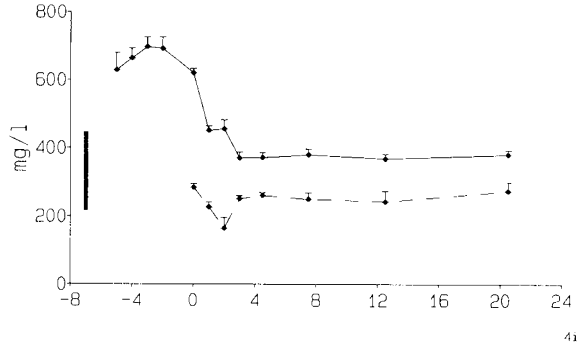

Fig. 2. Vitamin D binding protein, mean ± SEM, in plasma of mothers (•——•) and children (•---•) *vs.* time in wk (0 = d of delivery). Vertical bar: reference range, mean ± 2 SD.

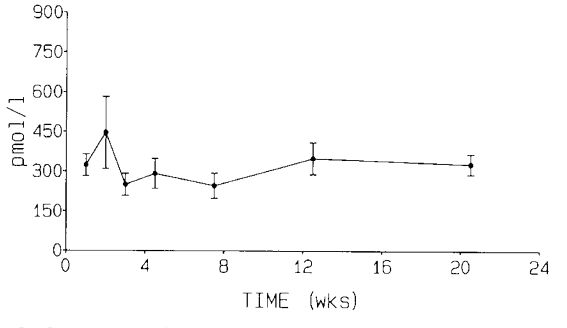

Fig. 3. 25-hydroxyvitamin D in breast milk, mean ± SEM, *vs.* time in wk.

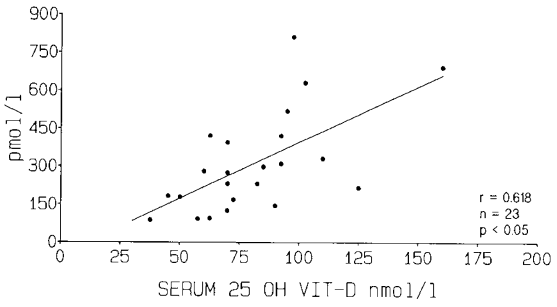

Fig. 4. Correlation between maternal plasma and breast milk concentrations of 25-hydroxyvitamin D, 1 wk after delivery.

1,25(OH)$_2$D could be detected (mean value, ~24 pmol/mL). In breast milk subjected to enzymatic hydrolysis by means of glucuronidase, the presence of glucuronides of 25OHD and 1,25(OH)$_2$D could not be detected.

DISCUSSION

Vitamin D metabolites in plasma. The group of mothers we studied delivered at the end of summer. High plasma 25OHD levels in the mothers were therefore expected. Maternal plasma values of 25OHD, 24,25(OH)$_2$D and 25,26(OH)$_2$D before delivery were within the adult reference range, whereas 1,25(OH)$_2$D levels were about twice the normal mean. This is in agreement with data in the literature. During pregnancy, unaltered or slightly decreased values were reported for 25OHD (10, 11) and for 24,25(OH)$_2$D (12). High maternal 1,25(OH)$_2$D values during pregnancy and at term are well documented (10–12). At birth the four vitamin D metabolites were significantly lower in cord than in maternal plasma. In contrast with total vitamin D metabolite concentrations, free 25OHD, 24,25(OH)$_2$D, and 25,26(OH)$_2$D levels were higher in cord than in maternal plasmas, but free 1,25(OH)$_2$D levels were virtually identical in maternal and cord plasma. Maternal DBP plasma levels, which are mainly under estrogen control, decrease sharply after birth. Free maternal 1,25(OH)$_2$D levels were halved within 1 wk.

In our study, maternal postpartum 24,25(OH)$_2$D and 25,26(OH)$_2$D levels followed the 25OHD pattern, which, taking into account the mean date of delivery, is in good agreement with seasonal variations as described for The Netherlands (13). Total 1,25(OH)$_2$D in infants rose sharply shortly after birth. Decreasing DBP levels in the 2nd wk led to a 5-fold increase in free 1,25(OH)$_2$D. High 1,25(OH)$_2$D levels reported on the 4th d of life (14) are in good agreement with our observation of a sharp rise of 1,25(OH)$_2$D after the 1st wk. One study reported an increase as early as 24 h after delivery (15). This increase of 1,25(OH)$_2$D and the even stronger increase of free 1,25(OH)$_2$D may be a physiologic response to the cut-off in the mineral supply via the placenta.

Towards the end of the study, plasma 24,25(OH)$_2$D levels decreased rapidly, reaching almost undetectable levels, whereas the correlation with 25OHD disappeared after the 3rd wk. The biochemical function of 24,25(OH)$_2$D in early childhood (if any) remains obscure. It might, however, be used as an "indicator" of the vitamin D stores (see below). Free 25,26(OH)$_2$D levels may be involved in maintaining the fetomaternal calcium gradient. Using multiple linear regression analysis, up to 40% of this gradient could be explained by fetal PTH levels and the difference between fetal and maternal free 25,26(OH)$_2$D (manuscript in preparation). The levels of 25,26(OH)$_2$D at birth were tightly correlated with the substrate 25OHD. After birth, the levels remained relatively constant, but the correlation with 25OHD levels disappeared. Compared to adults, in whom 25,26(OH)$_2$D synthesis seems to be dependent upon the availability of the

precursor 25OHD, the situation in neonates becomes distinctly different after the 1st weeks of life.

Placental transport. The most remarkable finding is that free $1,25(OH)_2D$ concentration in mothers equals the concentration observed in children. Experiments have demonstrated the transport of vitamin D (metabolites) from mother to fetus (16). Net placental transport was more rapid for 25OHD than for vitamin D in ewes (17) and more rapid for $1,25(OH)_2D$ than for 25OHD in human placentas (18). In a pregnant vitamin D receptor deficient patient, receiving very high doses of $1,25(OH)_2D$, maternal and cord levels of total $1,25(OH)_2D$ were found to be high and identical (19).

It seems reasonable that analogous transport mechanisms (active transport or passive diffusion) are involved for all four vitamin D metabolites. One could speculate that only free metabolites can cross the placenta and that free $1,25(OH)_2D$ determines the set point for the DBP level in the fetus. The fact that levels of free 25OHD, free $24,25(OH)_2D$, and free $25,26(OH)_2D$ are higher in cord than in maternal plasma is compatible with active transport against a gradient for these metabolites.

Vitamin D metabolites in milk. Total antirachitic activity in milk of nonvitamin D-supplemented lactating mothers is low (15–50 U/liter) (20, 21) and vitamin D sulfate levels were undetectable (22). Besides being sulfated, steroids and sterols are also often excreted conjugated with glucuronic acid. Although water-soluble vitamin D glucuronides were indeed reported to occur in the bile of rats (23) and of chickens (24), we have demonstrated that these metabolites do not contribute to the antirachitic activity in human breastmilk.

In milk, we found a mean 25OHD level of 325 pmol/liter. This is in good agreement with previous data (21, 25). Reported levels depend upon methodology, geographic site, ethnic origin, and food fortification. Values of $1,25(OH)_2D$ milk in our study were 3.4 pmol/liter. This is also in agreement with literature values (20, 21). Our data confirm a positive correlation between levels of 25OHD in maternal plasma and breast milk (Fig. 4), as found by Hollis *et al.* (25). A relationship between vitamin D intake and 25OHD levels in breast milk was found by Specker *et al.* (26).

Calcium absorption mechanisms in the newborn. Intestinal calcium absorption in the newborn is not solely under control of $1,25(OH)_2D$. Alkaline phosphatase for example can act as a calcium transporting protein (27). Lactose, amply available in breast milk, also may facilitate intestinal calcium absorption in the newborn. We think $1,25(OH)_2D$ has a function in early childhood. At birth, maternal and fetal free $1,25(OH)_2D$ levels are accurately attuned to each other, probably for the determination of the setpoint for DBP levels in the fetus. After birth, the infant's $1,25(OH)_2D$ rises sharply, which can be explained as a stimulus for the intestine to absorb calcium. Furthermore, hypocalcemic newborns respond to $1,25(OH)_2D$ therapy. However, the fact that only high-dose therapy is effective is circumstantial evidence that calcium absorption is not under $1,25(OH)_2D$ control only. Last but not least, strong evidence for $1,25(OH)_2D$-independent mechanisms is provided by two conditions in which $1,25(OH)_2D$ cannot exert its function: 1α-hydroxylase deficiency and $1,25(OH)_2D$ receptor deficiency. Affected patients manage to tide over a period of at least 6 mo until the first clinical signs occur (28, 29). The existence of $1,25(OH)_2D$ independent calcium absorption mechanisms may explain the lack of correlation between $1,25(OH)_2D$ and other clinical chemical parameters in early childhood. With aging the active ($1,25(OH)_2D$ dependent) processes seem to become more important (30).

Vitamin D supplementation. With regard to seasonal influences, the timing of this study was optimal. Mothers delivered at the end of the summer and had the full benefit of effective solar radiation resulting in high 25OHD plasma levels. However, in the children after the 1st mo some warning signs of vitamin D depletion became detectable. After one month 25OHD levels in some children were later found to have reached undesirably low levels and around the 8th wk 70% of the children had plasma 25OHD levels below the 5th adult reference percentile. Indeed, exclusively breast-fed infants showed a gradual reduction of bone mineral content up to the 12th wk of life when compared with infants receiving breast milk plus vitamin D supplementation or formula feeding (31). Assuming $24,25(OH)_2D$ is an inactive metabolite of 25OHD, the low vanishing levels of the former might be additional evidence for the depletion of vitamin D stores, favoring the biosynthesis of the active metabolite $1,25(OH)_2D$.

At approximately 8 wk, in 50% of the children, a moderately elevated phosphatase level was also observed (mean value of the "high phosphatase group" was 610 U/liter; upper reference value 500 U/liter).

In most European countries, breast feeding is common. In the Scandinavian countries, over 50% of infants at the age of 3 mo are still exclusively breastfed; the overall percentage for Europe exceeds 20% (32). In the Netherlands, this figure is about 30% (33). In the Netherlands, babies are not given vitamin D before the 3rd mo of life. The same is probably the case in several other countries. Our findings suggest that vitamin D supplementation, regardless the season of birth, should be started in the 1st or the 2nd wk of life.

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