Serum Concentration of 24, 25-Dihydroxyvitamin D in Normal Children and in Children with Rickets

THI MINH NGUYEN, H. GUILLOZO, M. GARABEDIAN, E. MALLET, AND S. BALSAN

*Laboratoire des Tissus Calcifiés (CNRS ER 126 and INSERM U.30) Hôpital des Enfants Malades, Paris, France
and Unité d'Explorations Fonctionnelles, Hôpital Charles Nicolles, Rouen, France*

Summary

The present method for the measurement of 24,25-dihydroxyvitamin D (24,25-(OH)$_2$D) in serum associates the highly sensitive competitive protein binding assay of Preece *et al.* and a double-step chromatography of the serum lipid extract (Sephadex LH 20 column and high pressure liquid chromatograph). With this assay, values observed in 16 normal children, 10 adolescents and 5 neonates, were not significantly different from those found in 14 normal adults (25-(OH)D: 20 ± 7.4 ng/ml, 24,25-(OH)$_2$D: 1.4 ± 0.8 ng/ml). No correlation was found between 25-(OH)D and 24,25-(OH)$_2$D serum concentrations. Measurements of 25-(OH)D and 24,25-(OH)$_2$D serum concentrations in some pathologic states demonstrated the existence of a 25-(OH)D-24-hydroxylase in children with three types of vitamin D resistant rickets (hereditary hypophosphatemia, pseudodeficiency rickets, and vitamin D resistance associated with Recklinghausen's neurofibromatosis). Finally, results observed during vitamin D administration suggest a regulation of the 24,25-(OH)$_2$D concentration in human serum: 1) 24,25-(OH)$_2$D serum concentration increased after vitamin D administration, yet it was not found higher in a 3-wk-old child with vitamin D$_2$ intoxication (25-(OH)D: 900 ng/ml, 24,25-(OH)$_2$D: 6 ng/ml) than in three children with vitamin D deficiency rickets in the 2nd wk after administration of vitamin D$_2$ and calcium (25-(OH)D: 25.5 ± 9.2 ng/ml, 24,25-(OH)$_2$D: 13.4 ± 3.7 ng/ml; 2) In a child with pseudodeficiency rickets, 24,25-(OH)$_2$D concentrations were found elevated before treatment. They decreased to normal values during treatment with 1α-(OH) cholecalciferol, whereas 25-(OH)D concentrations were not significantly different before and after treatment.

Speculation

Development of methods for the measurement of 24,25-(OH)$_2$D concentration in serum should provide valuable informations for a better understanding of vitamin D metabolism in physiologic and pathologic states in children.

One of the polar metabolites of 25-hydroxyvitamin D$_3$ (25-(OH)D$_3$), namely 24,25-dihydroxyvitamin D$_3$ (24,25-(OH)$_2$D$_3$) was first identified in 1973 (5). This metabolite is quantitatively the main 25-(OH)D$_3$ dihydroxyderivative found in the serum of normal animals (1, 3) and of normal human subjects (6). Its significance in vitamin D physiology is not clear (2, 3). Methods for the measurement of 24,25-(OH)$_2$D concentrations in human serum have been recently developed (4, 8, 10–12). The data obtained with these technics may help for a better understanding of the role of this metabolite. Results published so far concern mainly adults and, to our knowledge, only one investigation on serum 24,25-(OH)$_2$D$_3$ concentrations in neonates, children, and adolescents, has been reported (11). In 1976, an assay for 24,25-(OH)$_2$D measurement in lipid extracts of serum was developed in our laboratory. It includes a chromatography on Sephadex LH 20 column, a further purification step using a high pressure liquid chromatographic system and a competitive protein binding assay.

The present paper describes the method of 24,25-(OH)$_2$D measurement in serum and reports the results obtained in normal children as compared to normal adults; in children with vitamin D deficiency or vitamin D resistant rickets; and in one child intoxicated with ergocalciferol.

MATERIAL AND METHODS

VITAMIN D METABOLITES

[26,27-^{3}H]25-(OH)D$_3$ was purchased from the radiochemical Centre Amersham, Buckinghamshire, England (specific activity 11.7 or 12.1 Ci/mmole).

[26,27-^{3}H]24,25-(OH)$_2$D$_3$ was obtained from *in vitro* incubations of prepubertal rabbit kidney homogenates with tritiated 25-(OH)D$_3$, using the technique described for the production of 1,25-(OH)$_2$D$_3$ in chick kidney homogenates (7). After methanol-chloroform extraction of the kidney tissue, the chloroform phase was chromatographed on a 2 × 60 cm Sephadex LH 20 column equilibrated with chloroform-hexane 65:35 (v/v). 24,25-(OH)$_2$D$_3$ was then chromatographed on a high pressure liquid chromatograph as described later in this paper. Periodate sensitivity of ^{3}H-24,25-(OH)$_2$D$_3$ was verified before its use.

25-(OH)D$_3$ was a gift from Roussel Laboratory, Paris, France and 24R,25-(OH)$_2$D$_3$ a gift from Dr. Uskokovic, Hoffman-La Roche, Nutley, New Jersey. Both vitamin D metabolites were stored at −20°C in ethanol.

BINDING PROTEIN

Weanling rats were kept in a dark room and raised on a vitamin D deficient diet containing 0.47% calcium and 0.3% phosphorus. After 4–6 wk, rats were sacrificed by decapitation, the serum was collected and frozen in 10 μl aliquots at −20°C. A 1/20.000 (v/v) solution of this D-deficient rats serum in barbital acetate buffer pH 8.6 was used for the assays (9).

SERA EXTRACTION AND CHROMATOGRAPHY

Five hundred dpm of ^{3}H-25-(OH)D$_3$ and 500 dpm of ^{3}H-24,25-(OH)$_2$D$_3$ were added to 2 ml of serum samples before their extraction. After ½ hr at room temperature, chloroform-methanol extraction was performed as described by Preece et al. (9). The lipid extract was dissolved in 0.3 ml of 50:50 chloroform-n hexane and chromatographed on a Sephadex LH 20 column 185 × 5 mm equilibrated with the same solvent mixture. Thirty fractions of 1 ml were collected. A 0.2 ml aliquot of each fraction was dried down and the radioactivity counted in 10 ml of counting solution (5 g of PPO and 0.1 g of dimethyl POPOP/liter of toluene). Fractions in the ^{3}H-25-(OH)D$_3$ region (2–5 ml) and in the ^{3}H-24,25-(OH)$_2$D$_3$ region (7–13 ml) were pooled separately and dried down. The fraction containing 25-(OH)D$_3$ was applied on a silicic acid column to separate 25-(OH)D$_3$ from vitamin D and from metabolites less polar than 25-(OH)D$_3$ (9). The 24,25-(OH)$_2$D$_3$ fraction was dissolved in 0.1 ml ethanol and injected in a high pressure chromatograph (Waters Associates, Milford, Massachu-

setts 01757, USA) equipped with a solvent delivery system model 6000 A and a sample injector model V6K, fitted with a 4 mm I.D. × 30 cm μBondapak C₁₈ column. A linear gradient from 50–100% methanol in water was used as solvent (2.5% v/v per min) with a flow rate of 3 ml/min. Six ml fractions of the effluent were collected. An aliquot (0.5 ml) of each fraction was counted in 10 ml Instagel solution. The 24,25-(OH)₂D₃ region from high pressure liquid chromatography and the 25-(OH)D₃ region from silicic acid chromatography were dried down and dissolved in 2 ml ethanol. An aliquot of each of the final solutions was counted in order to measure the total recovery after the extraction and chromatographic procedures. Recovery was calculated as the ratio of the radioactivity present in the 2 ml ethanol solution, to the radioactivity added to the serum sample before the extraction. Mean percent of recovery for the dosages of 78 different blood samples was 73.1 ± 3.4 for 25-(OH)D and 53.4 ± 1.3 for 24,25-(OH)₂D.

The association of Sephadex LH 20 and high pressure liquid chromatographies allows a good separation of 24,25-(OH)₂D₃ from 25-(OH)D₃ and 1,25-(OH)₂D₃, as verified with tritiated metabolites. Synthetic metabolites of ergocalciferol were not available to investigate the ability of the chromatographic system to resolve vitamin D₂ and D₃ metabolites. Due to the size of the fractions isolated, it is most likely that 25-(OH)D and 24,25-(OH)₂D fractions contained the cholecalciferol and the ergocalciferol metabolites. Serum values are therefore expressed as ng/ml of 25-(OH)D and 24,25-(OH)₂D.

25-(OH)D COMPETITIVE BINDING ASSAY

25-(OH)D concentration was estimated with the assay described by Preece *et al.* (9). The high sensitivity of this assay allows the measurement of 25-(OH)D concentrations as low as 0.5 ng/ml of serum (0.02 pmole per incubation tube).

24,25-(OH)₂D COMPETITIVE BINDING ASSAY

It has been shown that 24,25-(OH)₂D₃ has the same affinity as 25-(OH)D₃ for the 25-(OH)D rat serum binding protein (4, 8, 10, 11). In our hands also, synthetic 24,25-(OH)₂D₃ was found as effective as 25-(OH)D₃ in displacing ³H-25-(OH)D₃ from its binding to the D-deficient rat serum protein. The 24,25-(OH)₂D concentration in serum was therefore measured with the 25-(OH)D competitive binding assay system *i.e.*, using as a standard curve, the displacement of ³H-25-(OH)D₃.

One serum was extracted ten times and each of the ten extractions was assayed twice for vitamin D metabolites. Within assay variations were, respectively, 9.3% for 25-(OH)D (11.8 ± 1.1 ng/ml) and 10.4% for 24,25-(OH)₂D (2.6 ± 0.27 ng/ml).

RESULTS

NORMAL SUBJECTS

Serum 25-(OH)D and 24,25-(OH)₂D concentrations were measured in 5 neonates, 16 children, and 10 adolescents with no alteration in phosphorus and/or calcium metabolism and no known renal, intestinal, or endocrine diseases. The values observed in these young subjects were compared to those found in 14 normal adults, aged 18–50 yr (Table 1). All samples were collected randomly throughout the year 1976.

In normal adults, serum concentrations of 25-(OH)D and 24,25-(OH)₂D (mean ± 1 SD) were 20 ± 7.4 and 1.4 ± 0.8 ng/ml, respectively. The concentrations of these metabolites (mean ± 1 SD) in children and adolescents were not significantly different from the values found in adults. In the five neonates studied before the 6th day of life neither 25-(OH)D concentrations nor 24,25-(OH)₂D concentrations were statistically different from the values found in older children or in adults.

When all the data were pooled and analyzed according to the seasonal period of sampling (Table 1), lower concentrations of serum 25-(OH)D were noted during the winter and spring 1976. These low concentrations in serum 25-(OH)D were associated with higher concentrations of 24,25-(OH)₂D. However, none of

these differences are statistically significant, presumably because of the small number of subjects studied in each season.

The analysis of the 25-(OH)D and 24,25-(OH)₂D serum values for the whole group of normal subjects studied did not demonstrate a positive correlation between serum concentrations of 24,25-(OH)₂D and 25-(OH)D (Fig. 1). It is to be noted that the seven highest values of 24,25-(OH)₂D serum concentrations were found in subjects with low 25-(OH)D serum concentrations (lower or equal to 12 ng/ml).

VITAMIN D-DEFICIENCY RICKETS

Serum 25-(OH)D and 24,25-(OH)₂D concentrations were measured in three children (aged 14–18 months) with overt radiologic signs of rickets increased serum alkaline phosphatase activities (respectively, 2470, 1850, and 1000 mU/ml of serum with normal values from 112–696 mU/ml), low serum calcium concentrations (respectively, 8.0, 7.8, and 8.8 mg/100 ml) and low serum phosphorus concentrations (respectively, 4.0, 3.1, and 2.7 mg/100 ml). Blood samples for vitamin D measurements could only be obtained after 1 wk of hospitalisation and after the start of oral calcium supplementation (180–360 mg/d). At that time, 25-(OH)D and 24,25-(OH)₂D concentrations were low, but not significantly different from the values found in normal children (Table 2). During the first 2 weeks after one im injection of 5 mg of vitamin D₂, the measurement of vitamin D metabolites showed a slight increase (basal value × 2.6) in serum 25-(OH)D concentration contrasting with a rapid and marked increase (basal value × 33)

Table 1. *Serum 25—(OH)D and 24,25—(OH)₂D concentrations (ng/ml) in normal subjects[1]*

	25—(OH)D	24,25—(OH)₂D
In relation to age		
<6 days (5)	13 ± 4.2	0.8 ± 0.4
2–10 yr (16)	15 ± 10.4	2.5 ± 1.9
14–17 yr (10)	14 ± 6.7	1.5 ± 0.9
Adult (over 18 yr) (14)	20 ± 7.4	1.4 ± 0.8
In relation to season		
January–March (14)	15 ± 9.1	2.2 ± 1.8
April–June (7)	11 ± 5.8	2.3 ± 1.1
July–September (5)	21 ± 7.4	1.2 ± 0.4
October–December (14)	19 ± 7.8	1.4 ± 0.7

[1] All values are expressed as mean ± SD. Numbers in parentheses are numbers of different blood samples.
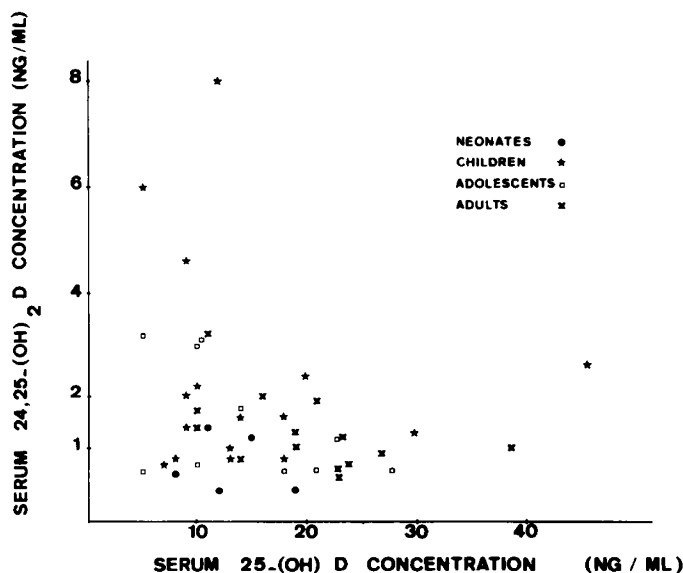

Fig. 1. Relationship between serum 24,25-(OH)₂D concentrations and serum 25-(OH)D concentrations in normal subjects. On this figure are pooled data from 5 neonates, 16 children, 10 adolescents, and 14 adults.

in 24,25-(OH)$_2$D concentration. The ratio of serum 24,25-(OH)$_2$D/ 25-(OH)D concentrations increased from 4% before treatment to 52% in the 2nd wk after vitamin D and calcium administration.

VITAMIN D INTOXICATION

In a 3-wk-old child referred with severe symptoms of vitamin D$_2$ intoxication, serum calcium concentrations had reached 14.0 mg/100 ml. Her serum 25-(OH)D concentration was 900 ng/ml and 24,25-(OH)$_2$D concentration 6 ng/ml. The serum 24,25-(OH)$_2$D/25-(OH)D ratio was 0.67%.

VITAMIN D RESISTANT RICKETS

Table 3 shows the data concerning three children with "pseudo-deficiency rickets" aged 11-16 yr, three subjects with hereditary hypophosphatemia aged 3-13 yr, and one patient with a vitamin D resistant rickets associated with Recklinghausen's neurofibromatosis. Pretreatment levels of 25-(OH)D and 24,25-(OH)$_2$D could be measured for three patients. The presence of 24,25-(OH)$_2$D was detected in the serum of the three patients (two with pseudo-deficiency rickets and one with hereditary hypophosphatemia). After 3 months to 5 yr of oral 25-(OH)D$_3$ administration serum 25-(OH)D concentrations were elevated in all patients. In contrast, serum 24,25-(OH)$_2$D concentrations were high in only three subjects (Table 3, patients K.A., S.K., and L.A.). These high values of 24,25-(OH)$_2$D were associated with the highest values of 25-(OH)D observed in this study, in two patients. In the third patient, it was associated with a moderately increased concentration of 25-(OH)D, i.e., 98 ng/ml. No correlation was found between serum 24,25-(OH)$_2$D concentrations and serum calcium and phosphorus concentrations (Table 3).

In a fourth child with pseudo-deficiency rickets, 25-(OH)D and 24,25-(OH)$_2$D serum concentrations were measured, before and during treatment with 1α-hydroxycholecalciferol (1α-(OH)D$_3$),

over a period of 4 yr and 6 months (Fig. 2). Serum 25-(OH)D concentrations were found most of the time in the normal range. Variations in 24,25-(OH)$_2$D concentrations could not be correlated with changes in 25-(OH)D concentrations. Higher than normal values of 24,25-(OH)$_2$D were observed before and in the first months after administration of the 1α-(OH)D$_3$. Serum 24,25-(OH)$_2$D concentrations were found in the normal range when biochemical and radiologic signs of rickets had disappeared. Higher than normal values were again observed when treatment had been stopped.

DISCUSSION

Recently developed methods for the measurement of 24,25-(OH)$_2$D are based on the similar potencies of 24,25-(OH)$_2$D$_3$ and 25-(OH)D$_3$ in displacing 25-(OH)D$_3$ from its binding sites in rat serum or kidney cell proteins (4, 8, 10, 11, 12). 25-(OH)D competitive protein binding assays are equally sensitive for the 25-(OH)D, 24,25-(OH)$_2$, 25,26-(OH)$_2$ metabolites of cholecalciferol (4, 8, 10, 11) and of ergocalciferol (9). Therefore, the chromatography of the serum is a crucial prerequisite to the measurement of vitamin D metabolites. In previous methods, 24,25-(OH)$_2$D was separated from 25-(OH)D on silica columns (8) or Sephadex LH 20 columns (4, 10, 11, 12). This last chromatographic technique also separates 24,25-(OH)$_2$D from 25,26-(OH)$_2$D. All authors used a competitive protein binding assay for the measurement of 25-(OH)D and 24,25-(OH)$_2$D concentrations in serum. The smallest quantities of 24,25-(OH)$_2$D (or 25-(OH)D) detected per assay tube vary from 0.01 pmole (8, 9) and 0.09 pmole (11), to 1.2-1.5 pmole (4, 10).

The present method associates a highly sensitive assay (9) and a double step chromatography of the serum. The Sephadex LH 20 plus high pressure liquid chromatography system was aimed to avoid contamination of the 24,25-(OH)$_2$D fraction by other vi-

Table 2. *Serum concentrations of 25—(OH)D, 24,25—(OH)$_2$D, calcium (Ca$_s$), and phosphorus (Pi$_s$), in three children with vitamin D deficiency rickets[1]*

	25—(OH)D (ng/ml)	24,25—(OH)$_2$D (ng/ml)	Ca$_s$ (mg/dl)	Pi$_s$ (mg/dl)
D deficiency rickets				
Before treatment	9.6 ± 1.2[2]	0.4 ± 0.2[2]	8.8 ± 0.5[2]	3.8 ± 0.9[2]
First wk of treatment	18.1 ± 4..4[2]	4.2 ± 4.3[2]	9.3 ± 0.5[2]	4.2 ± 1.6[2]
Second wk of treatment	25.5 ± 9.2[3]	13.4 ± 3.7[3]	9.6 ± 0.5[3]	5.1 ± 0.4[3]

[1] All three children with overt radiologic signs of rickets received calcium supplementation before vitamin D treatment. 25—(OH)D, 24,25—(OH)$_2$D, calcium, and phosphorus were measured 2-6 days before and in the 2 wk after one single im injection of 5 mg vitamin D$_2$.

[2] Mean ± 1 SD of values measured in six blood samples (two samples for each child).

[3] Mean ± 1 SD of values measured in three blood samples (one sample for each child).

Table 3. *Serum 25—(OH)D and 24,25—(OH)$_2$D concentrations (ng/ml) and corresponding serum calcium (Ca$_s$) and phosphorus (Pi$_s$) concentrations (mg/dl) in children with vitamin D resistant rickets*

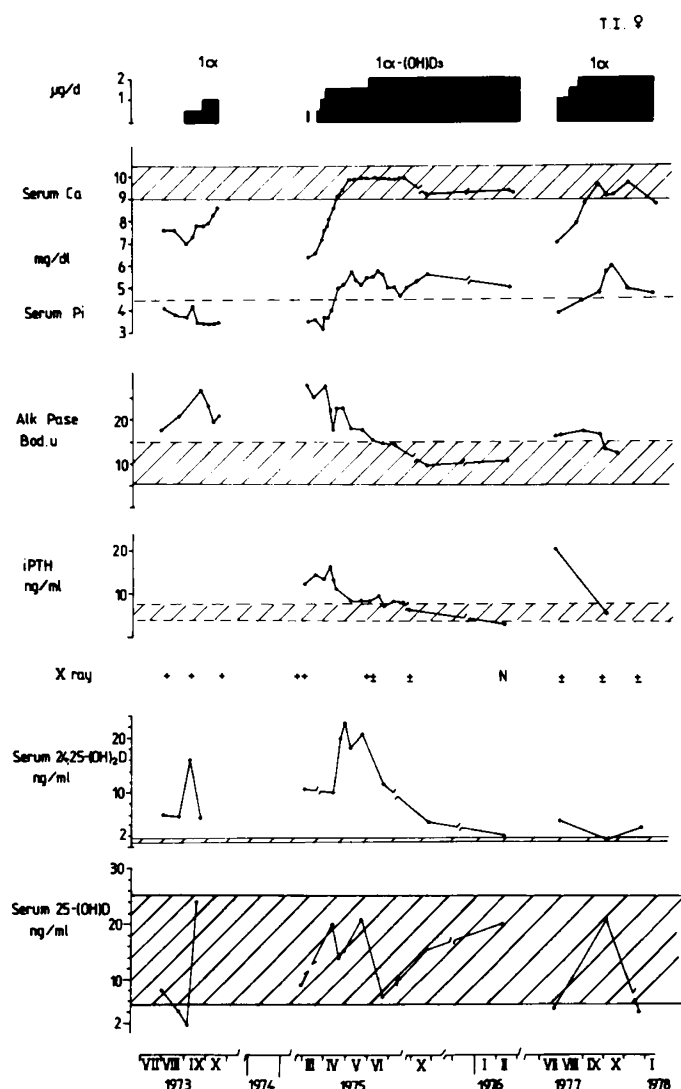|		Untreated				During treatment with 25—(OH)D$_3$			
	Age (yr)	25—(OH)D	24,25—(OH)$_2$D	Ca$_s$	Pi$_s$	25—(OH)D	24,25—(OH)$_2$D	Ca$_s$	Pi$_s$
Pseudodeficiency rickets									
K.S.	11					48	2.4	7.8	5.5
K.L.	16	14	1.6	5.2	4.6				
K.A.	14	24	2.5	6.9	3.9	90	2.0	6.3	4.2
						285	9.0	9.4	4.0
						128	0.8	9.5	3.4
						119	2.0	8.7	3.9
Hereditary hypophosphatemia									
S.K.	13	12	1.4	9.9	1.9	424	8.2	10.0	2.5
G.M.	13					61	2.8	9.2	2.1
L.A.	3					98	7.7	10.2	2.9
Vitamin D resistant rickets and neurofibromatosis									
L.J	20					142	2.6	9.0	2.4

Fig. 2. Evolution of biochemical and radiologic parameters before and during administration of 1α-(OH) cholecalciferol (0.5–2.0 μg/day) to a child with pseudodeficiency rickets. Serum calcium (Ca), and phosphorus (Pi) concentrations in mg/dl, serum alkaline phosphatase activities (Alk Pase) in Bodansky units (Bod. u.), serum immunoreactive parathyroid hormone concentrations (iPTH) in ng/ml, serum 24,25-(OH)₂D and 25-(OH)D concentrations in ng/ml. Radiograms were normal (N) or showed slight (±) to moderate (++) signs of rickets. *Hatched areas* represent for each parameter the range of normal values. The child was 7-yr-old at the beginning of the study.

tamin D metabolites, especially when studying subjects on large vitamin D therapy. With this technique, the serum 24,25-(OH)₂D concentrations found in normal French adults are similar to those found by Taylor *et al.* (10) (1.68 ± 0.8 ng/ml), by O'Riordan *et al.* (8) (1.7 ng/ml) in England, and by Caldas *et al.* (12) (4.3 ± 2.0 nmole/liter *i.e.*, 1.78 ± 0.8 ng/ml) in United States. They are lower than those observed in North America by Haddad *et al.* (4).

Our values in normal children are not significantly different from those found in normal adults. At the present time, there is only one report of serum 24,25-(OH)₂D concentrations in children. These values are 3.3 ± 1.3 ng/ml for 24,25-(OH)₂D and 35.2 ± 9.2 ng/ml for 25-(OH)D (11). Our data in neonates, although not statistically significant, could be in agreement with the suggestion that 24,25-(OH)₂D serum concentrations are lower in neonates than in older children (11).

A positive correlation between serum 25-(OH)D and 24,25-

(OH)₂D concentrations in normal subjects (11, 12) and in subjects receiving vitamin D supplementation (8, 10) has been reported. In the present work, 24,25-(OH)₂D serum concentrations increased when 25-(OH)D levels were elevated by vitamin D administration to children with vitamin D deficiency rickets. Yet no positive correlation was found between the serum concentrations of these two vitamin D metabolites in normal adults or in normal children. Other observations from the present study show that 24,25-(OH)₂D serum concentration is not always correlated with 25-(OH)D serum concentration, and suggest that regulation of the 24,25-(OH)₂D concentration in human serum may exist: 1) after administration of high doses of vitamin D or 25-(OH)D, higher concentrations of 24,25-(OH)₂D in serum were not necessarily associated with greater values of serum 25-(OH)D. A striking example is the finding of high 24,25-(OH)₂D concentrations (13.4 ± 3.7 ng/ml) a few days after vitamin D₂ administration to children with vitamin D deficiency rickets (25-(OH)D serum concentration of 25.5 ± 9.2 ng/ml) and of similarly high 24,25-(OH)₂D concentration (6 ng/ml) in a child with vitamin D₂ intoxication (25-(OH)D concentration of 900 ng/ml). 2) in a child with pseudodeficiency rickets, treatment of the rachitic lesions with 1α-(OH)D₃ was accompanied by changes in 24,25-(OH)₂D serum concentration which could not be explained by changes in 25-(OH)D concentration.

Finally, development of methods for the measurements of 24,25-(OH)₂D concentration in serum should provide valuable informations for the comprehension of some pathologic states. Thus, the present results demonstrate the existence of a 25-(OH)D-24-hydroxylase in children with three types of vitamin D resistant rickets (hereditary hypophosphatemia, pseudodeficiency rickets, and vitamin D resistance associated with Recklinghausen's neurofibromatosis).

REFERENCES AND NOTES

1. Boyle, I. T., Gray, R. W., and DeLuca, H. F.: Regulation by calcium of *in vivo* synthesis of 1,25-dihydroxycholecalciferol and 21,25-dihydroxycholecalciferol. Proc. Nat. Acad. Sci. USA, *68:* 2131 (1971)
2. Boyle, I. T., Omdahl, J. L., Gray, R. W., and DeLuca, H. F.: The biological activity and metabolism of 24,25-dihydroxyvitamin D₃. J. Biol. Chem., *25:* 4174 (1973)
3. DeLuca, H. F.: Recent advances in our understanding of the vitamin D endocrine system. J. Lab. Clin. Med., *87:* 7 (1976)
4. Haddad, J. G., Min, C., Walgate, J., and Hahn, T.: Competition by 24,25-dihydroxycholecalciferol in the competitive protein binding radioassay of 25-hydroxycalciferol. J. Clin. Endocrinol. Metab., *43:* 712 (1976)
5. Holick, M. F., Schnoes, H. K., and DeLuca, H. F.: Isolation and identification of 24,25-dihydroxycholecalciferol: a metabolite of vitamin D₃ made in the kidney. Biochemistry, *11:* 4251 (1972)
6. Mawer, E. B., Backhouse, J., Hill, L. F., Lumb, G. A., De Silva, P., Taylor, C. M., and Stanbury, S. W.: Vitamin D metabolism and parathyroid function in man. Clin. Sci. Molec. Med., *48:* 349 (1975)
7. Omdahl, J. L., Gray, R. W., Boyle, I. T., Knutson, J., and DeLuca, H. F.: Regulation of metabolism of 25-hydroxycholecalciferol by kidney tissue *in vitro* by dietary calcium. Nature (New Biol.), *237:* 63 (1972)
8. O'Riordan, J. L. H., Graham, R. F., and Dolev, E.: An assay for 24,25 and 25,26-dihydroxycholecalciferol in sera. In: Vitamin D, Biochemical, Chemical, and Clinical Aspects Related to Calcium Metabolism. Proceedings of the Third Workshop on Vitamin D, p. 519 (Walter de Gruyter, Berlin, New York, 1977)
9. Preece, M. A., O'Riordan, J. L. H., Lawson, D. E. M., and Kodicek, E.: A competitive protein-binding assay for 25-hydroxycholecalciferol and 25-hydroxyergocalciferol in serum. Clin. Chim. Acta, *54:* 235 (1974)
10. Taylor, C. M., Hughes, S. E., and Da Silva, P.: Competitive protein binding assay for 24,25-dihydroxycholecalciferol. Biochem. Biophys. Res. Comm., *70:* 1243 (1976)
11. Weisman, Y., Reiter, E., and Root, A.: Measurement of 24,25-dihydroxyvitamin D in sera of neonates and children. J. Pediatr., *91:* 904 (1977)
12. Caldas, A. E., Gray, R. W., and Lemann, J., Jr.: The simultaneous measurement of vitamin D metabolites in plasma: studies in healthy adults and in patients with calcium nephrolithiasis. J. Lab. Clin. Med., *91:* 840 (1978)
13. The authors thank Dr. J. L. H. O'Riordan (London, U.K.) for helpful discussions that led to the development of their 24,25-(OH)₂D₃ assay. Our grateful thanks, also, to Dr. M. Uskokovic (Hoffman-La Roche, Nutley, New Jersey, USA), and the Roussel Laboratory (Paris, France) for providing the synthetic vitamin D metabolites.
14. This research was supported, in part, by grant 77 7 0668 from the D.G.R.S.T.
15. Received for publication March 28, 1978.
16. Accepted for publication August 30, 1978.