Impaired 24,25-Dihydroxyvitamin D Production in Anephric Human and Pig

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ABSTRACT Plasma 25-hydroxyvitamin D and 24, 25-dihydroxy-vitamin D [24,25-$(OH)_2D$] concentrations were measured in normal and chronically dialyzed anephric humans and pigs. Measurement of the 24, 25-$(OH)_2D$ was preceded by three purification steps involving one Sephadex LH-20 column and two high-pressure liquid chromatographic columns. The final high-pressure liquid chromatography step involved resolution of 25-hydroxy-vitamin D_3-26,23 lactone and 25,26-dihydroxy-vitamin D_2 from 24,25-dihydroxyvitamin D_2 and 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$]. The total 25-hydroxyvitamin D [25-hydroxyvitamin D_2 plus 25-hydroxyvitamin D_3 (25-OHD_3)] was 31.7±3.6 ng/ml in the plasma of eight anephric human subjects and 40.1±3.7 ng/ml in five normal human subjects. Six of the eight anephric patients had undetectable (<0.2 ng/ml) 24,25-$(OH)_2D$ concentrations. Two of the eight patients had very low (0.51 and 0.41 ng/ml), but detectable, 24,25-dihydroxyvitamin D_2. The normal human volunteers had plasma 24,25-$(OH)_2D$ concentrations of 2.8±0.7 ng/ml. Chronically dialyzed anephric and normal pigs were given intramuscular injections of massive amounts (5×10^6 IU) of vitamin D_3 immediately after surgery (day 0) and again on day 7. In anephric pigs, plasma 25-OHD_3 progressively rose from 12±4 ng/ml on day 0 to 705±62 ng/ml on day 10. The 25-OHD_3 concentrations in normal pigs rose from 8±2 ng/ml on day 0 to 439±64 ng/ml on day 10. Plasma 25-OHD_3 was higher in anephrics throughout the experiment, and concentrations were significantly higher ($P < 0.05$) on days 9 and 10. Plasma 24,25-$(OH)_2D_3$ concentrations declined progressively in anephric pigs from 3.6±0.6 ng/ml on day 0 to 3.2±0.7 ng/ml on day 2. During days 4–10, plasma 24,25-$(OH)_2D_3$ was not apparent until plasma 25-OHD_3 was >400 ng/ml. In control pigs, plasma 24,25-$(OH)_2D_3$ was elevated from 4.3±0.6 ng/ml on day 0 to 178±2.7 ng/ml on day 3. Plasma 24,25-$(OH)_2D_3$ was significantly higher (P <0.05) in controls on days 1–8. At the end of the experiment (day 10), 24,25-$(OH)_2D_3$ concentrations were similar and not significantly different in both groups (87.0±18.4 ng/ml in anephric and 110.3±32.1 ng/ml in normal pigs). The identity of the 24,25-$(OH)_2D_3$ isolated from anephric pig plasma was confirmed by mass spectroscopy. Our data suggest that anephric humans receiving normal dietary levels of vitamin D_3 have little or no ability to produce 24,25-$(OH)_2D$. However, we have shown that pigs produce 24,25-$(OH)_2D_3$ when plasma 25-OHD_3 is extremely high (>400 ng/ml).

INTRODUCTION

The role of the kidney in the formation of active vitamin D_3 metabolites has been shown in many laboratories (1–3). Nephrectomy stops bioproduction of 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$][1] (4) or the newly discovered vitamin D_3 metabolite, 25-hydroxyvitamin D_3-26,23 lactone (25-OHD_3-26,23 lactone) (5). However, there is still some question whether 24, 25-dihydroxyvitamin D [24,25-$(OH)_2D$] is exclusively a product of the kidney. Garabedian et al. (6) showed the production of a polar peak in anephric rats given large doses of [^{3}H]25-hydroxyvitamin D_3 ([^{3}H]-25-OHD_3). By means of the polar peak's periodate sensitivity and comigration with authentic 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$] on Sephadex

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[1] *Abbreviations used in this paper*: HPLC, high-pressure liquid chromatography; OHD, hydroxyvitamin D; $(OH)_2D$, dihydroxyvitamin D; 25-OHD_3-26,23 lactone, 25-hydroxyvitamin D_3-26,23 lactone.

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LH-20, it was identified as 24,25-$(OH)_2D_3$. However, no mass spectra data were published to confirm their beliefs. Haddad et al. (7) recently reported concentrations of 3.0 ng/ml of 24,25-$(OH)_2D$ in both anephric and normal humans. Horst et al. (8) and Shepard et al. (9) reported 24,25-$(OH)_2D$ at concentrations of 0.8 and 1.9 ng/ml, respectively, in anephric human plasma. In all three studies normal 25-hydroxyvitamin D (25-OHD) concentrations (15–40 ng/ml) were observed in normal subjects and anephric patients. In contrast to the measurable 24,25-$(OH)_2D$ found by some workers (7–9), Taylor (10) failed to detect 24,25-$(OH)_2D$ in plasma of anephric man with normal or above normal 25-OHD concentrations. These conflicting reports are undoubtedly due to the different ways of measuring 24,25-$(OH)_2D_3$ in plasma-lipid extracts. Information concerning 24,25-$(OH)_2D$ concentrations (or production) in anephric patients is important because its presence may be required for the function of 1,25-$(OH)_2D_3$ in the normal healing of osteomalacia in humans (11).

The results of this report show that the amount of 24,25-$(OH)_2D$ is very low to nondetectable in anephric human plasma. However, we will show that anephric pigs produce 24,25-$(OH)_2D_3$ when plasma 25-OHD_3 concentrations are elevated to >400 ng/ml by massive intramuscular injections of vitamin D_3.

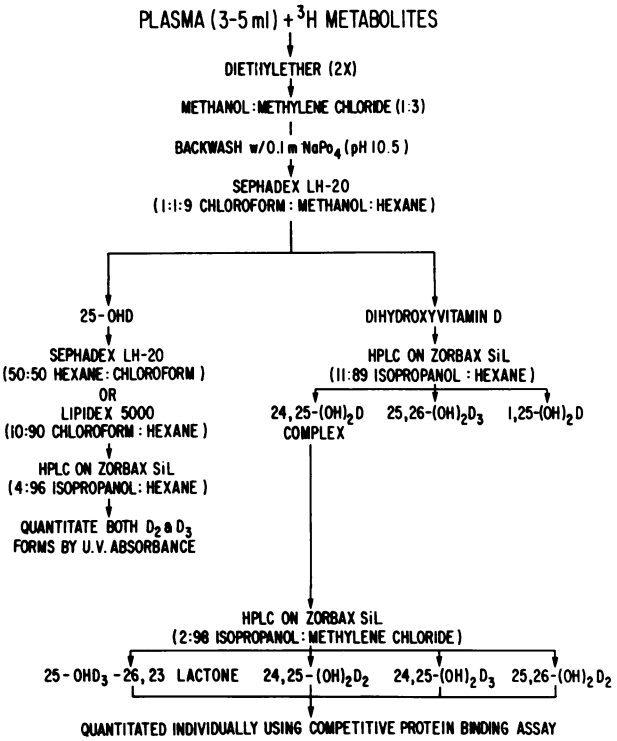

FIGURE 1 Procedure used for the isolation and measurment of 25-OHD_2, 25-OHD_3, 24,25-$(OH)_2D_2$, and 24,25-$(OH)_2D_3$.

METHODS

Sterols and assays. 25-OHD_3 was a gift from the Upjohn Company, Kalamazoo, Mich. Vitamin D_3 was purchased from Sigma Chemical Co., St. Louis, Mo. 24,25-$(OH)_2D_3$ was a gift from Hoffman-La Roche, Nutley, N. J. 24,25-Dihydroxyvitamin D_2 [24,25-$(OH)_2D_2$] on 25,26-dihydroxyvitamin D_2 [25,26-$(OH)_2D_2$] were isolated and identified from vitamin D_2-toxic plasma.[2] Concentrations of all metabolites used as standards (originating from vitamin D_2 or D_3) were measured in 100% ETOH (E_{max} = 18,200 ~264 nm) with a Beckman DB scanning spectrophotometer (Beckmann Instruments, Fullerton, Calif.).

The procedure for the purification of the 24,25-$(OH)_2D$ and 25-OHD for assay is shown in Fig. 1. Briefly, plasma lipids were removed by extracting plasma twice with 3 vol peroxide-free diethyl ether and once with 4 vol 1:3 MeOH:$MeCl_2$. The lipid extracts were purified for measurement of 25-OHD and 24,25-$(OH)_2D$ by a modification of the procedure of Horst et al. (8). They proposed a method of quantify 24,25-$(OH)_2D$ and 25,26-$(OH)_2D$ by radioligand binding assay after purification by high-pressure liquid chromatography (HPLC) on a Zorbax Sil column (Dupont Instruments, Wilmington, Del.) developed in 11:89 isopropanol:hexane. We have found that the 24,25-$(OH)_2D$ region of this column contains not only 24,25-$(OH)_2D_2$ and 24,25-$(OH)_2D_3$, but also 25,26-$(OH)_2D_2$ and 25-OHD_3-26,23 lactone (Fig. 2). In addition, Horst et al. (8) found material that competed in the binding assay that migrated very near the 24,25-$(OH)_2D$ region of this column. This material, designated as peak I, was five- to sixfold higher in anephric plasma, and its presence was independent of vitamin D (7). These substances were separated when the 24,25-$(OH)_2D$ region from the first HPLC Zorbax Sil column was chromatographed on another HPLC Zorbax Sil column developed in 2:98 isopropanol:methylene chloride. The 24,25-$(OH)_2D_2$ and 24,25-$(OH)_2D_3$ regions (Fig. 3) were collected and measured by radioligand binding assays. The radioligand binding assays (8) were modified by adding 0.01% gelatin to the rat plasma, diluted (1/5,000) in 0.05 M $NaPO_4$ buffer (pH 7.5). The addition of the gelatin resulted in higher specific binding than was obtained with diluted plasma alone and in little change in nonspecific binding (Fig. 4). No [^{3}H]24,25-$(OH)_2D_2$ was available for estimating the recovery of this metabolite, so we assumed a recovery similar to that of 24,25-$(OH)_2D_3$ because identical purification steps were used for the measurement of both 24,25-$(OH)_2D_2$ and 24,25-$(OH)_2D_3$. A typical standard curve for each metabolite is shown in Fig. 5.

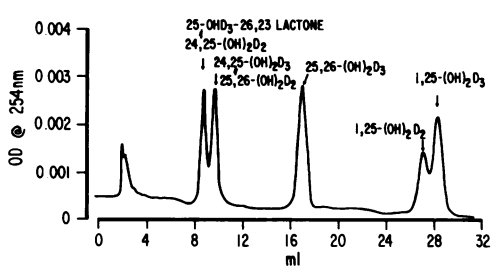

FIGURE 2 Elution of vitamin D_2 and vitamin D_3 metabolites from an HPLC Zorbax Sil column developed in 11:89 isopropranol:hexane with a flow rate of 2.0 ml/min.

[2] Horst, R. L., E. T. Littledike, J. L. Riley, and J. L. Napoli. Manuscript submitted for publication.

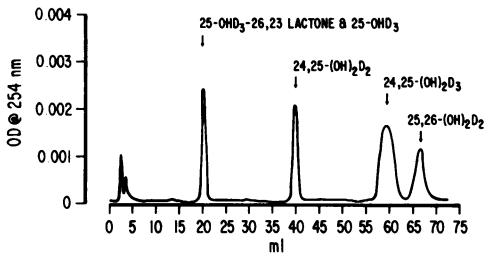

FIGURE 3 Elution of vitamin D_2 and vitamin D_3 metabolites from an HPLC Zorbax Sil column developed in 2:98 isopropanol:methylene chloride with a flow rate of 2.0 ml/min.

Human blood samples. Blood samples were obtained from normal human volunteers and anephric patients who had been bilaterally nephrectomized at least 1 yr before our experiment. The samples for both groups were taken during August and September of 1979. In addition to their usual dietary sources, the anephric patients (with the exception of A-8) received 1,000 IU/d of vitamin D_2 orally.

Pig blood samples. Bilateral nephrectomy (anephrics) or sham-operation (controls) were performed on 5- to 6-wk-old pigs (3 pigs/group) fed a diet containing normal concentrations (2,200 IU/kg of diet) of vitamin D_3. On the day of surgery (day 0) and again on day 7, the pigs were treated intramuscularly with 5×10^6 IU to vitamin D_3. All pigs, both anephrics and controls, were subjected to peritoneal dialysis twice daily for 45 min with 500 ml of Ringer's solution that was modified to contain 5–10% dextrose. The dialysis solution was introduced and removed through a chronically implanted catheter placed in the peritoneal cavity. Blood samples from these pigs were taken frequently (as shown in Fig. 6).

Identity of 24,25-$(OH)_2D_3$ generated in vivo in anephric pigs. At the end of the experiment the anephric pigs were bled. The plasma lipids were extracted as described (8). 24,25-$(OH)_2D_3$ was isolated and identified by mass spectroscopy; a Varian CH-7 mass spectrometer (Varian Associates, Palo Alto, Calif.) was used at 70 eV with a direct probe inlet at 90°C above ambient temperature.

RESULTS

Vitamin D metabolites in human plasma. No 24, 25-$(OH)_2D_3$ was detected ($\leq$0.2 ng-ml) in the plasma of eight anephric humans (Table I). 24,25-$(OH)_2D_2$ was detected in the plasma of two anephric patients, A-5 (0.51 ng/ml) and A-7 (0.41 ng/ml). Plasma 24,25-$(OH)_2D_3$, however, was detected (overall mean±SE, 2.3±0.42) and was the major circulating form of 24,25-$(OH)_2D$ in normal patients. Plasma 24,25-$(OH)_2D_2$ was not detected in three of the five normal humans and was very low in the other two normal humans (Table I).

Plasma 25-OHD concentrations were presented and summarized in Table I. 25-OHD concentrations were 31.7±3.6 (mean±SE) in anephrics and 40.1±3.7 (mean±SE) in normals. The contribution of 25-hydroxyvitamin D_2 (25-OHD_2) to the total plasma 25-OHD pool ranged from 5.3–60% in anephrics and 3–5% in normals.

Vitamin D_3 metabolites in pig plasma. No vitamin D_2 metabolites were detected in pig plasma; therefore, only the metabolites originating from vitamin D_3 will be reported.

The longitudinal changes in plasma 25-OHD_3, 24,25-$(OH)_2D_3$, and calcium are shown in Table II and

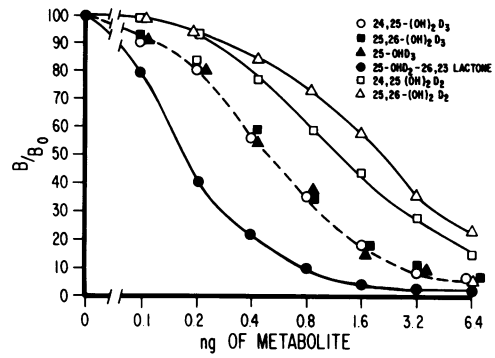

FIGURE 5 Relative binding of vitamin D_2 and vitamin D_3 metabolites in the competitive protein binding assay for 24,25-$(OH)_2D$.

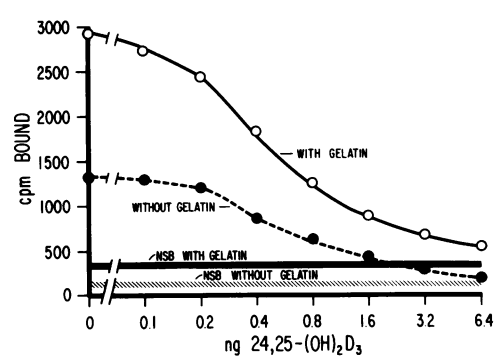

FIGURE 4 Influence of the addition of gelatin to the competitive protein binding assay for 24,25-$(OH)_2D_3$. The y axis represents the counts per minute [^{3}H]25-OHD_3 bound. NSB, nonspecific binding.

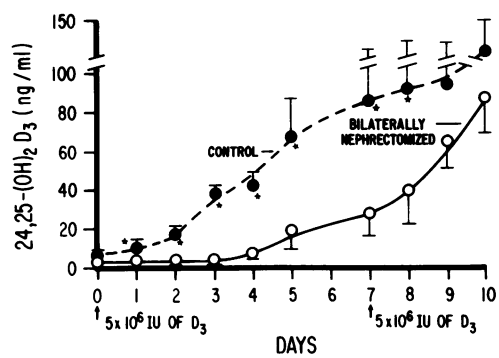

FIGURE 6 Changes in plasma 24,25-$(OH)_2D_3$ in anephric (bilaterally nephrectomized) and control pigs after intramuscular injections of vitamin D_3 (5×10^6 IU) on day O and again on day 7. *, $P < 0.05$.

TABLE I

25-OHD$_2$, 25-OHD$_3$, 24,25-(OH)$_2$D$_2$, and 24,25-(OH)$_2$D$_3$ in Normal and Anephric Human

Group	Metabolite					
	25-OHD$_2$	25-OHD$_3$	Total 25-OHD	24,25-(OH)$_2$D$_2$*	24,25-(OH)$_2$D$_3$*	Total 24,25-(OH)$_2$D
			ng/ml			
Anephric						
A-1	25.6	16.2	41.8	<0.2	<0.2	ND
A-2	12.8	13.4	26.2	<0.2	<0.2	ND
A-3	15.3	32.1	47.4	<0.2	<0.2	ND
A-4	6.8	17.9	24.7	<0.2	<0.2	ND
A-5	10.7	10.0	20.7	0.51	<0.2	0.51
A-6	10.8	25.6	36.4	<0.2	<0.2	ND
A-7	11.7	8.5	20.2	0.41	<0.2	0.41
A-8	2.0	35.7	37.7	<0.2	<0.2	ND
Mean±SE	11.8±2.3	19.9±3.6	31.7±3.6	0.26±0.12	<0.2	0.12±0.08
Normal						
N-1	2.2	38.3	40.5	0.32	4.9	5.2
N-2	2.4	31.4	33.8	0.36	1.5	1.9
N-3	4.5	30.4	34.9	<0.2	1.4	1.4
N-4	1.7	35.8	37.5	<0.2	2.2	2.2
N-5	3.7	50.2	53.9	<0.2	3.2	3.2
Mean±SE	2.9±0.5	37.2±3.6	40.1±3.7	0.26±0.07	2.6±0.42	2.8±0.70

* In samples with <0.2 ng/ml of 24,25-(OH)$_2$D, a value of 0.2 was used in calculating the mean.

in Figs. 6 and 7. On day 0, just before surgery and the first vitamin D$_3$ injection, the plasma 24,25-(OH)$_2$D$_3$ was 4.3±0.61 ng/ml in the control pigs and 3.6±0.6 ng/ml in the anephric pigs. In the anephric pigs, the plasma 24,25-(OH)$_2$D$_3$ declined slightly by day 2 to 3.2 ng/ml; however, on day 4 the plasma 24,25-(OH)$_2$D$_3$ began rising progressively to 87.0±18.4 ng/ml by day 10. In control pigs, the plasma 24,25-(OH)$_2$D$_3$ more than doubled from 4.3±0.6 to 9.8±4.1 ng/ml by day 1 and progressively increased to 110.3±32.1 ng/ml by day 10. Plasma 24,25-(OH)$_2$D$_3$ concentrations in control pigs were higher than anephrics ($P < 0.05$) from day 1 to day 8. To positively identify the material measured as 24,25-(OH)$_2$D$_3$, we isolated ~2 μg of 24,25-(OH)$_2$D$_3$ from 50 ml of pooled plasma from anephric pigs taken on day 10. The mass spectra (Fig. 8) confirmed the material as 24,25-(OH)$_2$D$_3$. The molecular ion at the ratio of mass to charge (m/e) 416 and the peaks at (398

TABLE II

Changes in 25-OHD$_3$, 24,25-(OH)$_2$D$_3$, and Calcium (Ca) in Three Anephric and Three Control Pigs

Day	Anephric			Controls		
	25-OHD$_3$	24,25-(OH)$_2$D$_3$	Ca	25-OHD$_3$	24,25-(OH)$_2$D$_3$	Ca
	ng/ml		*mg/dl*	*ng/ml*		*mg/dl*
0	12±4	3.6±0.6	8.4±0.1	8±2	4.3±0.6	10.3±1.2
1	60±19	2.8±0.7	8.8±0.5	64±11	9.8±4.1	10.1±0.3
2	267±94	3.2±0.7	13.7±0.7	181±17	17.8±2.7	10.1±0.8
3	341±120	4.6±0.9	15.5±0.1	212±14	27.5±5.6	10.9±0.9
4	422±126	7.5±2.0	16.2±0.2	332±65	41.5±8.6	10.7±0.6
5	—	—	—	—	—	—
6	433±86	19.1±9.5	13.3±0.5	339±93	66.7±21.8	10.9±0.1
7	656±74	26.7±11.7	12.4±0.2	438±42	83.9±32.4	10.8±0.7
8	576±96	39.2±17.9	12.7±0.4	388±11	89.8±31.4	11.6±0.4
9	758±80	65.4±14.3	12.0±0.1	367±24	92.9±28.4	10.7±1.1
10	705±62	87.0±18.4	11.7±0.2	439±64	110.3±32.1	—

After intramuscular injections of 5 × 10^6 IU of vitamin D$_3$ on day 0 and day 7. Data are given as the mean±SE.

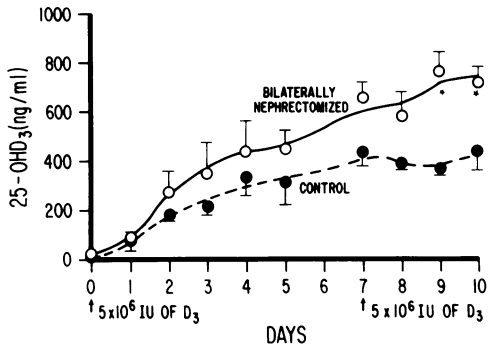

FIGURE 7 Changes in 25-OHD_3 in anephric (bilaterally nephrectomized) and control pigs treated as described in Fig. 6. *, $P < 0.05$.

M^+-H_2O), 383 (M^+-H_2O-CH_3), 271 (M^+-side chain), 253 (271-H_2O), 136 (A ring plus carbons 6 and 7), and 118 (136-H_2O) are consistent with the mass fragmentation of authentic 24,25-$(OH)_2D_3$ (12).

The plasma 25-OHD_3 concentrations in anephric and normal pigs are shown in Table II and Fig. 7. All the pigs had 25-OHD_3 concentrations ranging from 7 to 15 ng/ml on day 0 just before surgery. By day 1, 25-OHD_3 concentrations in both anephrics and controls had risen to 50-70 ng/ml. By day 10 (3 d after the second vitamin D_3 injection), the anephric pigs had significantly higher ($P < 0.05$) concentrations (705±62 ng/ml) of 25-OHD_3 than control (439±64 ng/ml).

Plasma-Ca concentrations remained normal or slightly below (8–10 mg/dl) during days 0, 1, and 2 in both anephric and control pigs. Thereafter, a transient hypercalcemia was observed in the anephric pigs; Ca concentration (16.2 mg/dl) was highest on day 4. Plasma Ca was slightly elevated in normals during days 8–10.

DISCUSSION

Our assay techniques, which involved an extension of purification procedures previously described (8), resulted in the demonstrations of very low to non-detectable levels of 24,25-$(OH)_2D$ in anephric human plasma. This result is in contrast to those in previous reports (7–9), in which detectable plasma 24,25-$(OH)_2D$ in anephric human plasma samples are described. Although none of the eight anephric patients in the present report had detectable plasma 24,25-$(OH)_2D_3$, two anephric patients (A-5 and A-7) had very low but detectable 24,25-$(OH)_2D_2$. Plasma 25-OHD_2 concentrations in these two patients (10.7 and 11.7 ng/ml) were similar to those in most of the other anephric patients. The reason for the measurable 24,25-$(OH)_2D_2$ in these two patients is unknown.

Our results, therefore, confirm those of Taylor (10) who showed that extremely low or unmeasurable plasma 24,25-$(OH)_2D$ concentrations exist in anephric humans consuming normal dietary levels of vitamin D. This final resolution of whether 24,25-$(OH)_2D$ is produced in anephrics is very important because a great deal of experimental evidence suggests that 1,25-dihydroxyvitamin D [1,25-$(OH)_2D$] is the only active hormonal form of vitamin D and is the only metabolite not produced in anephric humans (4). Although results in early studies suggest that 1,25-$(OH)_2D$ may be adequate for treating children with vitamin D deficiency (13), results in several other studies are in direct conflict with this concept (11, 14). In diseases in which plasma 24,25-$(OH)_2D$ and 1,25-$(OH)_2D$ concentrations would be very low (vitamin D deficiency and renal osteodystrophy), treatment with 1,25-$(OH)_2D_3$ alone did not heal the osteomalacia associated with these diseases (11, 14). However,

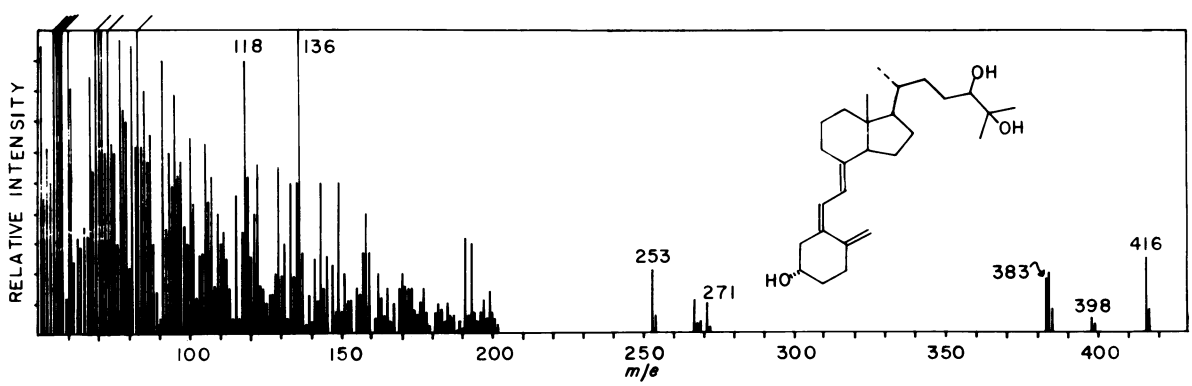

FIGURE 8 Mass spectrum of 24,25-$(OH)_2D_3$ isolated from anephric pigs. The 24,25-$(OH)_2D_3$ was isolated from plasma taken on day 10 of the experiment. The spectrum shows an average of two scans. The molecular ion at the ratio of mass to charge (m/e) 416 and the peaks at m/e 398 (M^+-H_2O), 383 (M^+-H_2O-CH_3), 271 (M^+-side chain), 253 (271-H_2O), 136 (A ring plus carbons 6 and 7), and 118 (136-H_2O) clearly shows that the compound is a dihydroxylated vitamin D_3 derivative. The peaks at m/e 271, 253, 136, and 118 further show that the two additional hydroxyl groups are in the side chain.

when 24,25-$(OH)_2D_3$ was given in addition to 1,25-$(OH)_2D_3$, the resulting normal bone mineralization in patients with vitamin D-deficient osteomalacia (11, 14) suggested an active role for 24,25-$(OH)_2D_3$ in normal bone formation. Although untested, response to this dual treatment might be similar in patients with renal osteodystrophy.

Another important aspect of this study is whether anephrics of any species have the ability to produce 24,25-$(OH)_2D$ in extrarenal tissues such as the intestine (15) or bone (16). Taylor (10) has shown that 24,25-$(OH)_2D$ was not detectable in anephric humans with normal 25-OHD concentrations (20–40 ng/ml) or in anephric subjects with two- to threefold normal 25-OHD concentrations. Therefore, we used the anephric and control pigs injected with massive amounts of vitamin D_3 to assure the achievement of superphysiological plasma 25-OHD_3 concentrations. In these experiments, the control pigs had elevated plasma 24,25-$(OH)_2D_3$ concentration within 24 h after the initial massive injection of vitamin D_3. In general, the plasma 24,25-$(OH)_2D_3$ concentrations in control pigs paralled the plasma 25-OHD_3 concentration so that by the end of the experiment the plasma 24,25-$(OH)_2D_3$ concentrations in control pigs had increased to 110 ng/ml. In contrast, the 24,25-$(OH)_2D_3$ concentrations in the anephrics had decreased slightly from presurgical concentrations of 3.6 to 2.8 ng/ml by day 1 and 3.2 ng/ml by day 2 (Fig. 6 and Table II). Plasma 24,25-$(OH)_2D_3$ in anephric pigs decreased during the same period in which plasma 25-OHD_3 concentrations were increasing. However, after day 4, the 24,25-$(OH)_2D_3$ concentrations in the plasma of the anephric pigs progressively increased to 87.0±18.4 ng/ml by day 10. Extrarenal 24-hydroxylation was first shown only when 25-OHD_3 concentrations had reached superphysiological concentrations of >400 ng/ml (Figs. 6 and 7). The reason for the inability of anephric pigs to 24-hydroxylate 25-OHD_3 when 25-OHD_3 is <400 ng/ml is unknown. One explanation may be that the K_m for the 25-OHD_3 of the extrarenal 24-hydroxylase might be higher than the kidney 24-hydroxylase, and thus require more substrate for hydroxylation. Alternatively, the extrarenal enzyme may require several days for activation or stimulation. The first of these hypotheses seems more likely because all patients in our study had been nephrectomized for at least 1 yr. Presumably, this would be enough time for extrarenal 24-hydroxylase stimulation. Therefore, attempting to cause an elevation in plasma 24,25-$(OH)_2D$ in anephric patients by giving exogenous vitamin D_3 or 25-OHD_3 might lead to toxic side effects from the high plasma concentrations of 25-OHD_3 needed to satisfy the substrate concentration requirements of the extrarenal 24-hydroxylase enzyme. The hypercalcemic state of anephric pigs after vitamin D_3 injections in our experiment (Table II) supports this concept.

Conclusion. We have confirmed earlier findings (10) that 24,25-$(OH)_2D$ is very low to nondetectable in anephric humans consuming normal dietary levels of vitamin D. This result conflicts with results in previous work (7–9). From our results, it is apparent that the other purification methods (7–9) for 24,25-$(OH)_2D$ determination do not adequately resolve material that will compete in the competitive protein-binding assay. We did not detect any 25,26-$(OH)_2D_2$ or 25-OHD_3-26,23 lactone in normal or anephric human plasma. Apparently, therefore, other compounds (possibly peak I in Horst et al. [8]) not related to vitamin D were measured as 24,25-$(OH)_2D$; previous results, therefore, are erroneous. Although our system for 24, 25-$(OH)_2D$ measurement is more laborious than that of Taylor (10), it has the advantage of being able to isolate and measure other metabolites of vitamin D_2 and vitamin D_3.

Finally, we have shown that anephric pigs produce 24,25-$(OH)_2D_3$ when given pharmacological doses of vitamin D_3 to cause superphysiological plasma 25-OHD_3 concentrations of >400 ng/ml.

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REFERENCES

1. Fraser, D. R., and E. Kodichet. 1970. Unique biosynthesis by kidney of a biologically active vitamin D metabolite. *Nature (Lond.).* **228:** 764–766.
2. Knutson, J. C., and H. F. DeLuca. 1974. 25-Hydroxyvitamin D_3-24-hydroxylase. Subcellular location and properties. *Biochemistry.* **13:** 1543–1548.
3. Tanaka, Y., R. A. Shepard, H. F. DeLuca, and H. K. Schnoes. 1978. The 26-hydroxylation of 25-hydroxyvitamin D_3 in vitro by chick renal homogenates. *Biochem. Biophys. Res. Commun.* **83:** 7–13.
4. DeLuca, H. F. 1979. The vitamin D system in the regulation of calcium and phosphorus metabolism. *Nutr. Rev.* **37:** 161–193.
5. Horst, R. L., and E. T. Littledike. 1980. 25-OHD_3-26,23 lactone: demonstration of kidney-dependent synthesis in the pig and rat. *Biochem. Biophys. Res. Commun.* **93:** 149–154.
6. Garabedian, M., H. Pavlovitch, C. Fellot, and S. Balsan. 1974. Metabolism of 25-hydroxyvitamin D_3 in anephric rats: a new active metabolite. *Proc. Natl. Acad. Sci. U. S. A.* **71:** 554–557.

7. Haddad, J. G., Jr., C. Min, M. Mendelsohn, E. Slatopolsky, and T. J. Hahn. 1977. Competitive protein-binding radioassay of 24,25-dihydroxyvitamin D in sera from normal and anephric subjects. *Arch. Biochem. Biophys.* **182:** 390–395.
8. Horst, R. L., R. M. Shepard, N. A. Jorgensen, and H. F. DeLuca. 1979. The determination of 24,25-dihydroxyvitamin D and 25,26-dihydroxyvitamin D in plasma from normal and nephrectomized man. *J. Lab. Clin. Med.* **93:** 277–285.
9. Shepard, R. M., R. L. Horst, A. J. Hamstra, and H. F. DeLuca. 1979. Determination of vitamin D and its metabolites in plasma from normal and anephric man. *Biochem. J.* **182:** 55–69.
10. Taylor, C. M. 1977. The measurement of 24,25-dihydrocholecalciferol in human serum. *In* Vitamin D: Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism. A. W. Norman, K. Schaefer, D. V. Herrath, H.-G. Grigoleit, J. W. Coburn, H. F. DeLuca, E. B. Mawer, T. Suda, editors. Walter De Gruyter, Berlin. 541–545.
11. Bordier, D., H. Rasmussen, P. Marie, L. Miravet, J. Gueris, and A. Ryckwaert. 1978. Vitamin D metabolites and bone mineralization in man. *J. Clin. Endocrinol. Metab.* **46:** 284–294.
12. 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 D_3 made in the kidney. *Biochemistry.* **11:** 4251–4255.
13. Balsan, S., M. Garabedian, R. Sorgniard, M. F. Holick, and H. F. DeLuca. 1975. 1,25-Dihydroxyvitamin D_3 and 1α-hydroxyvitamin D_3 in children: biological and therapeutic effects in nutritional rickets and different types of vitamin D resistance. *Pediatr. Res.* **9:** 586–597.
14. Bordier, P., J. Zingraff, J. Gueris, P. Jungers, P. Marie, M. Pechet, and H. Rasmussen. 1978. The effect of 1α-OHD_3 and 1α,25-$(OH)_2D_3$ on the bone in patients with renal osteodystrophy. *Am. J. Med.* **64:** 101–107.
15. Kumar, R., H. K. Schnoes, and H. F. DeLuca. 1978. Rat intestinal 25-hydroxyvitamin D_3 and 1α,25-dihydroxyvitamin D_3-24-hydroxylase. *J. Biol. Chem.* **253:** 3804–3809.
16. Garabedian, M., M. B. DeBois, M. T. Corval, E. Pezant, and S. Balsan. 1978. Vitamin D and cartilage. I. *In vitro* metabolism of 25-hydroxycholecalciferol by cartilage. *Endocrinology.* **102:** 1262–1268.