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Biochemistry

23,25-Dihydroxyvitamin D_3: A natural precursor in the biosynthesis of 25-hydroxyvitamin D_3-26,23-lactone*

(vitamin D metabolism/25-hydroxyvitamin D/calcium metabolism/phosphorus metabolism)

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ABSTRACT **To elucidate the biosynthesis of 25-hydroxyvitamin D_3-26,23-lactone, two known metabolites of 25-hydroxyvitamin D_3—23,25-dihydroxyvitamin D_3 and 25,26-dihydroxyvitamin D_3—were incubated individually with kidney homogenate prepared from vitamin D-supplemented chickens, a preparation known to produce the lactone from 25-hydroxyvitamin D_3. The 25-hydroxyvitamin D_3-26,23-lactone produced *in vitro* was then separated, purified, identified, and quantitated by consecutive straight-phase and reverse-phase high-performance liquid chromatography. 23,25-Dihydroxyvitamin D_3 is a far better substrate for production of 25-hydroxyvitamin D_3-26,23-lactone than is 25,26-dihydroxyvitamin D_3. Production of lactone is highly selective for the natural 23(*S*)-hydroxy-23,25-dihydroxyvitamin D_3 while both epimers of 25,26-dihydroxyvitamin D_3 resulted in small amounts of product comigrating with natural lactone. It appears that 23(*S*),25-dihydroxyvitamin D_3, but not 25,26-dihydroxyvitamin D_3, is a natural precursor in the synthesis of 25-hydroxyvitamin D_3-26,23-lactone; this result also implies that the configuration of the lactone at C-23 is *S*.**

One of the major metabolites of vitamin D_3 has recently been detected in the plasma of vitamin D-supplemented chickens (1) and in man (2), isolated in pure form, and identified as 25-hydroxyvitamin D_3-26,23-lactone (25-OH-D_3-26,23-lactone; ref. 3). This compound is not produced in nephrectomized rats, but kidney homogenate prepared from vitamin D-supplemented chickens produces it from 25-hydroxyvitamin D_3 (25-OH-D_3; refs. 4 and 5). Another metabolite of 25-OH-D_3 was also detected during these studies; its *in vivo* synthesis is also abolished by nephrectomy (6). This compound was isolated in pure form and identified by spectroscopic analysis and chemical synthesis as 23,25-dihydroxyvitamin D_3 [23,25-$(OH)_2D_3$; ref. 6]. Comparison with synthetic products of known stereochemistry has established the 23(*S*) configuration for this metabolite (7). Neither the natural metabolite nor its C-23 epimer show significant biological activity (unpublished results) and thus its biological function is unknown. Similarly, the physiological function of 25,26-dihydroxyvitamin D_3 [25,26-$(OH)_2D_3$] is not understood even though it has significant biological activity in animals (8) and is also produced by kidney (9). Both 25,26-$(OH)_2D_3$ and 23,25-$(OH)_2D_3$ are potential precursors in renal synthesis of 25-OH-D_3-26,23-lactone. Recently, Hollis *et al.* (10) claimed that 25,26-$(OH)_2D_3$ is the intermediate in the synthesis of 25-OH-D_3-26,23-lactone because it was superior to 25-OH-D_3 as substrate in this system. In contrast, Pramanik and Napoli (11) suggested that 25,26-$(OH)_2D_3$ might not be an intermediate in the synthesis of the lactone because no lactone was detected in plasma of rats administered 25,26-$(OH)_2D_3$. To find the biosynthetic pathway for 25-OH-D_3-26,23-lactone, we incubated kidney homogenate prepared from vitamin D-supplemented chickens with either 23(*S*),25-$(OH)_2D_3$, 23(*R*),25-$(OH)_2D_3$, 25(*R*),26-$(OH)_2D_3$, 25(*S*),26-$(OH)_2D_3$, or 25-OH-D_3. The 25-OH-D_3-26,23-lactone produced *in vitro* was then isolated, identified, and quantitated by consecutive straight-phase high-performance liquid chromatography (HPLC) and reverse-phase HPLC. The 23(*S*),25-$(OH)_2D_3$ was found to be the superior substrate for production of 25-OH-D_3-26,23-lactone, suggesting it to be the natural precursor in the synthesis of 25-OH-D_3-26,23-lactone.

MATERIALS AND METHODS

Vitamin D Compounds. 25-OH-D_3 was a gift from Upjohn, and 1,25-$(OH)_2D_3$, 25(*S*),26-$(OH)_2D_3$ and 25(*R*),26-$(OH)_2D_3$ were gifts from Hoffmann–La Roche. 23(*S*),25-$(OH)_2D_3$ and 23(*R*),25-$(OH)_2D_3$ were synthesized in our laboratory (7). 25-OH-D_3-26,23-Lactone was synthesized by Wichmann *et al.* (12). All other reagents were purchased from Sigma.

Animals. One-day-old single-comb White Leghorn chickens were purchased from Northern Hatcheries (Beaver Dam, WI) and fed a rachitogenic diet (13) for 1 month. For the final 3 days, they were given 5 μg of 25-OH-D_3 daily in 95% ethanol/propylene glycol (5:95) subcutaneously. They were also given 5 μg of 1,25-dihydroxyvitamin D_3 in the same manner 18 hr prior to sacrifice.

***In Vitro* Incubation of Chicken Kidney Homogenate.** A 20% (wt/vol) homogenate of kidney was prepared in ice-cold 15 mM Tris acetate (pH 7.4)/0.19 M sucrose. Incubation of 600 mg of tissue was carried out in 6 ml (final volume) of 15 mM Tris acetate, pH 7.4/0.19 M sucrose/22.4 mM glucose 6-phosphate/20 mM ATP/160 mM nicotinamide/0.4 mM NADP/5 mM $MgCl_2$/0.1 M KCl/glucose-6-phosphate-dehydrogenase (0.5 unit)/25 mM sodium succinate in a 125-ml Erlenmeyer flask. The reaction was initiated by the addition of 10 μg of 23(*S*),25-$(OH)_2D_3$, 23(*R*),25-$(OH)_2D_3$, 25(*S*),26-$(OH)_2D_3$, 25(*R*),26-$(OH)_2D_3$, or 25-OH-D_3 in 50 μl of 95% ethanol. Four reaction mixtures for each substrate, similarly prepared, were incubated at 37°C with shaking at 100 oscillations per min for 3 hr in an atmosphere of air. The contents of the four flasks were then pooled, and the resulting 24 ml of incubation medium was extracted with 120 ml of methylene chloride (14).

Purification of Extract for HPLC by Sephadex LH-20 Column. The methylene chloride extract was evaporated to dryness under reduced pressure, and the residue was dissolved in 1 ml

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Abbreviations: 25-OH-D_3-26;23-lactone, 25-hydroxyvitamin D_3-26,23-lactone; 25-OH-D_3, 25-hydroxyvitamin D_3; 23,25-$(OH)_2D_3$, 23,25-dihydroxyvitamin D_3; 25,26-$(OH)_2D_3$, 25,26-dihydroxyvitamin D_3; HPLC, high-performance liquid chromatography.

* No reprints available.

of 65% chloroform/hexane and applied to a Sephadex LH-20 column (0.7 cm × 14 cm) packed in the same solvent (15). The column was eluted with 30 ml of the same solvent. The first 8 ml of eluant containing lipid was discarded and the next 22 ml, which contained 25-OH-D_3-26,23-lactone and unchanged substrate, was collected (16).

Straight-Phase HPLC. The purified extract was dried under reduced pressure, and the residue was dissolved in 6% 2-propanol/hexane and subjected to HPLC (ALC/GPC 204 instrument with a 254-nm UV detector; Waters Associates) using a Zorbax-SIL column (4.6 mm × 25 cm) (Du Pont). HPLC was performed with 6% 2-propanol/hexane at 1000 lbf/in^2 (1 lbf/in^2 = 6.895 kPa) and 2 ml/min (Fig. 1). The elution positions of the metabolites were established by comparison with those of authentic compounds (Fig. 1). Fractions containing 25-OH-D_3-26,23-lactone were collected for further purification by reverse-phase HPLC.

Reverse-Phase HPLC. The fractions of 25-OH-D_3-26,23-lactone collected from straight-phase HPLC were dried, and the residues were dissolved in 22% H_2O/methanol for reverse-phase HPLC on a Lichrosorb RP-18 column (4.6 mm × 25 cm) (Merck, Darmstadt, Federal Republic of Germany). The column was eluted with 22% H_2O/methanol at 1300 lbf/in^2 and 2 ml/min. The elution position of 25-OH-D_3-26,23-lactone was established by comparison with that of the authentic compound (Fig. 2). Quantitation of the lactone produced *in vitro* was calculated at this stage while another reverse-phase column was used for cochromatography of biologically produced and authentic lactone. The putative 25-OH-D_3-26,23-lactone was collected from the first reverse-phase column and mixed with an equal amount of authentic 25-OH-D_3-26,23-lactone. In the case in which no compound eluted at the position of 25-OH-D_3-26,23-lactone (Fig. 2*B*), the compound that eluted just before the lactone was collected and mixed with authentic 25-OH-D_3-26,23-lactone. Cochromatography was performed as described above except that the solvent was 25% H_2O/methanol (Fig. 3), which provides superior resolution of those compounds (4). The elution position of 25-OH-D_3-26,23-lactone was determined by comparison with that of the authentic compound.

Quantitation of Metabolite Produced *in Vitro*. The area under the peak indicated by the UV detector was measured, and the amount of metabolite produced *in vitro* was calculated by comparison with a standard curve made by plotting the area under the peak indicated by the UV detector vs. the amount of authentic compound subjected to HPLC.

RESULTS

As shown in Fig. 1*A*, a substantial amount of compound was eluted at the elution position of 25-OH-D_3-26,23-lactone when 23(*S*),25-$(OH)_2D_3$ was incubated with kidney homogenate while no corresponding compound was produced from 23(*R*),25-$(OH)_2D_3$ (Fig. 1*B*). Large peaks at 23 ml in Fig. 1*A* and at 18 ml in Fig. 1*B* are unchanged substrates, 23(*S*),25-$(OH)_2D_3$ and 23(*R*),25-$(OH)_2D_3$, respectively. In contrast, a minute peak was detected at the elution position of the lactone when either 25(*S*),26-$(OH)_2D_3$ (Fig. 1*C*) or 25(*R*),26-$(OH)_2D_3$ (Fig. 1*D*) was incubated with the same kidney homogenate preparation. Large peaks at 50 ml in Fig. 1 *C* and *D* are unchanged substrates. The suspected 25-OH-D_3-26,23-lactone produced *in vitro* (Fig. 1 *A*, *C*, and *D*) was collected and further purified by reverse-phase HPLC using 22% H_2O/methanol as shown in Fig. 2. Although no 25-OH-D_3-26,23-lactone was produced from 23(*R*),25-$(OH)_2D_3$ (Fig. 1*B*), the unknown compound at

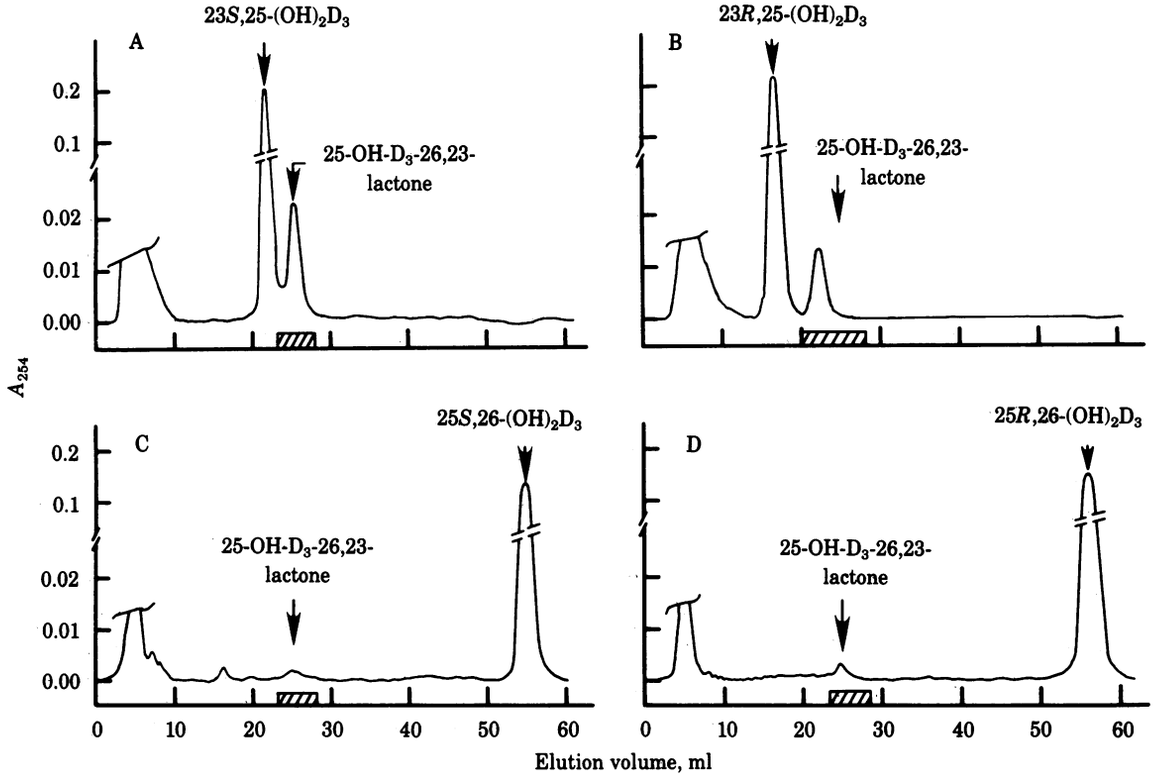

FIG. 1. HPLC of extracts of incubation mixtures using various vitamin D compounds and chicken kidney homogenates. Kidney homogenate prepared from vitamin D-supplemented chickens was incubated with 10 μg of 23(*S*),25-$(OH)_2D_3$ (*A*), 23(*R*),25-$(OH)_2D_3$ (*B*), 25(*S*),26-$(OH)_2D_3$ (*C*), or 25(*R*),26-$(OH)_2D_3$ (*D*). Arrows represent elution volumes of authentic compounds. Fractions containing 25-OH-D_3-26,23-lactone (▨) were collected for reverse-phase HPLC (Fig. 2).

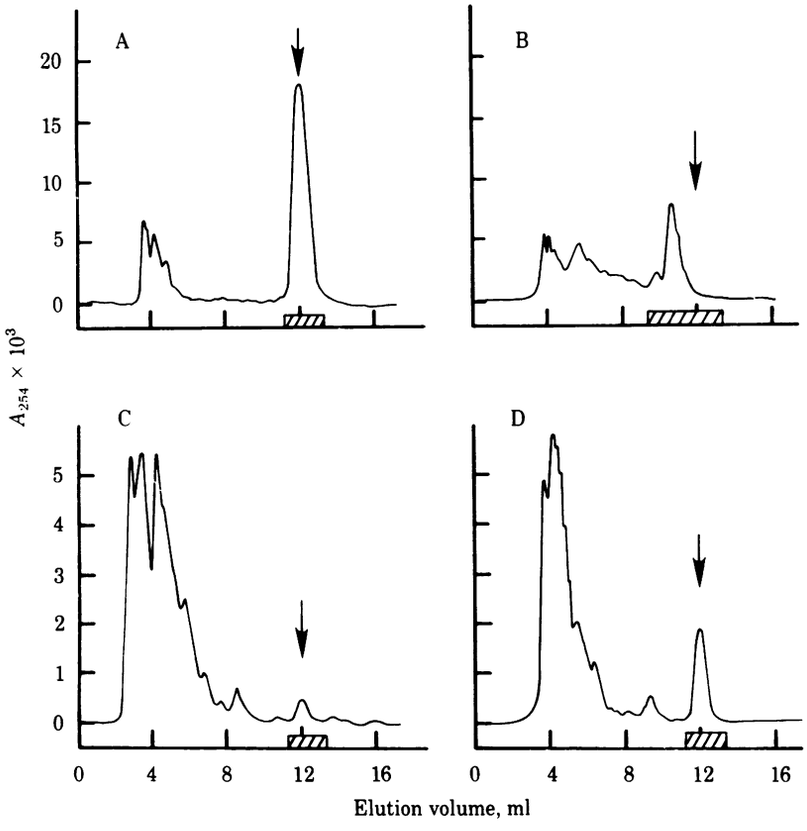

FIG. 2. Purification by reverse-phase HPLC of 25-OH-D_3-26,23-lactone produced as shown in Fig. 1. 23(*S*),25-$(OH)_2D_3$ (*A*), 23(*R*),25-$(OH)_2D_3$ (*B*), 25(*S*),26-$(OH)_2D_3$ (*C*), or 25(*R*),26-$(OH)_2D_3$ (*D*). Fractions collected from straight-phase HPLC were subjected to reverse-phase HPLC with 22% H_2O/methanol. Arrows indicate elution position of authentic 25-OH-D_3-26,23-lactone. Fractions containing 25-OH-D_3-26,23-lactone (▨) were collected for cochromatography with the authentic compound (Fig. 3).

22 ml was collected and subjected to reverse-phase HPLC to ensure that it was not 25-OH-D_3-26,23-lactone. The 25-OH-D_3-26,23-lactone produced *in vitro* and collected from straight-phase HPLC (Fig. 1) eluted exactly at the position of authentic 25-OH-D_3-26,23-lactone when the substrate was 23(*S*),25-$(OH)_2D_3$, 25(*S*),26-$(OH)_2D_3$, or 25(*R*),26-$(OH)_2D_3$ (Fig. 2 *A*, *C*, and *D*), while an unknown compound (Fig. 1*B*) eluted earlier than 25-OH-D_3-26,23-lactone (Fig. 2*B*). Purified 25-OH-D_3-26,23-lactone collected from the reverse-phase HPLC (Fig. 2 *A*, *C*, and *D*) and the unknown compound (Fig. 2*B*) were co-

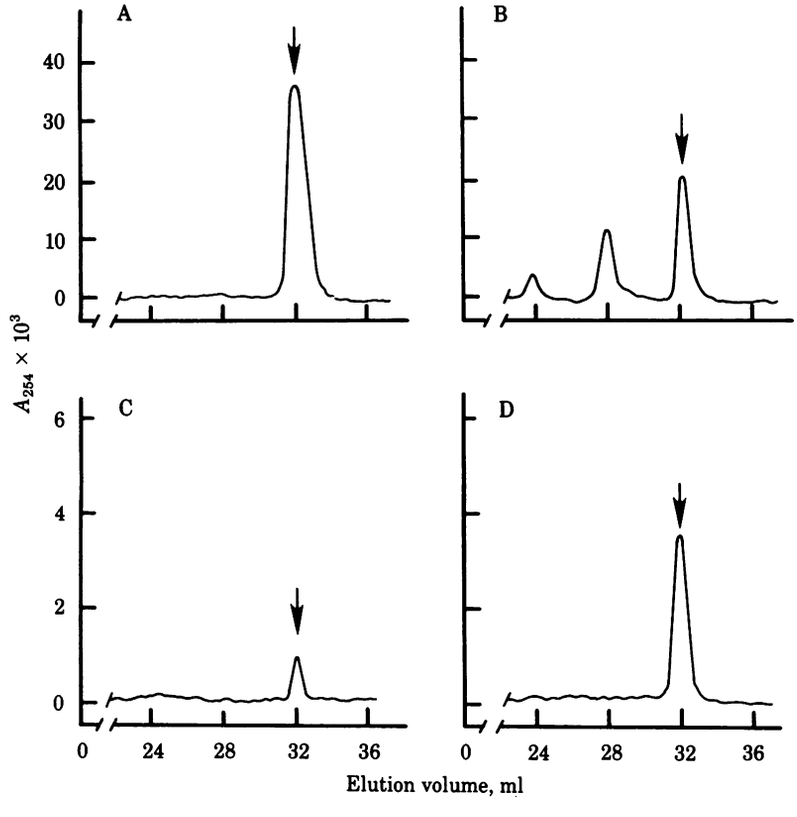

FIG. 3. Cochromatography of compound produced *in vitro* with authentic 25-OH-D_3-26,23-lactone on reverse-phase HPLC. The lactone produced *in vitro* from 23(*S*),25-$(OH)_2D_3$ (*A*), 25(*S*),26-$(OH)_2D_3$ (*C*), or 25(*R*),26-$(OH)_2D_3$ (*D*) and the compounds produced from 23(*R*),25-$(OH)_2D_3$ (*B*) were collected as shown in Fig. 2, mixed with an approximately equal amount of authentic 25-OH-D_3-26,23-lactone, and cochromatographed on reverse-phase HPLC using 25% H_2O/methanol. Arrows indicate elution position of authentic 25-OH-D_3-26,23-lactone.

Table 1. Production of 25-OH-D_3-26,23-lactone

Substrate	Lactone produced, (ng/2.4 g of tissue)/3 hr
23(*S*),25-$(OH)_2D_3$	430
23(*R*),25-$(OH)_2D_3$	ND*
25(*S*),26-$(OH)_2D_3$	12
25(*R*),26-$(OH)_2D_3$	54

A 20% kidney homogenate was incubated with 10 μg of substrate for 3 hr. 25-OH-D_3-26,23-lactone produced *in vitro* was isolated by straight-phase HPLC using 6% 2-propanol/hexane and purified by reverse-phase HPLC using 22% H_2O/methanol. The purified compound was quantitated by comparison with a standard curve made with authentic 25-OH-D_3-26,23-lactone.
* Not detectable (<5 ng).

Table 2. *In vitro* production of 23,25-$(OH)_2D_3$, 25,26-$(OH)_2D_3$, and 25-OH-D_3-26,23-lactone from 25-OH-D_3

Metabolite produced	Amount, (ng/2.4 g of tissue)/3 hr
23,25-$(OH)_2D_3$	144
25,26-$(OH)_2D_3$	32
25-OH-D_3-26,23-lactone	15

A 20% kidney homogenate was incubated with 10 μg of 25-OH-D_3 for 3 hr. Those metabolites produced *in vitro* were separated by straight-phase HPLC using 6% 2-propanol/hexane and purified by reverse-phase HPLC using 22% H_2O/methanol. Amounts of metabolites were quantitated by comparison with the corresponding standard curves. In this system, 24(*R*),25-$(OH)_2D_3$ is the major metabolite, but it was not quantitated in this study.

chromatographed with authentic 25-OH-D_3-26,23-lactone on reverse-phase HPLC using 25% H_2O/methanol as shown in Fig. 3. The 25-OH-D_3-26,23-lactone produced from 23(*S*),25-$(OH)_2D_3$, 25(*S*),26-$(OH)_2D_3$, or 25(*R*),26-$(OH)_2D_3$ (Fig. 3 *A*, *C*, and *D*) exactly comigrated with the authentic lactone, while the unknown peak (Fig. 2*B*) eluted at 24 ml and at 28 ml, separated from the authentic lactone (Fig. 3*B*). The amount of 25-OH-D_3-26,23-lactone produced *in vitro* from each substrate was calculated from the peak area shown in Fig. 2. The results are shown in Table 1. It was observed that there is a high degree of selectivity for production of 25-OH-D_3-26,23-lactone. 23(*S*),25-$(OH)_2D_3$ is clearly the superior substrate, while 25,26-$(OH)_2D_3$ is a poor precursor, and the naturally occurring 23(*R*),25-$(OH)_2D_3$ is the poorest substrate.

The metabolites produced when the kidney preparations were incubated with 25-OH-D_3 as substrate are given in Table 1. It is clear that 25-OH-D_3 is converted in large amounts to 23(*S*),25-$(OH)_2D_3$, in lesser amounts to 25(*S*),26-$(OH)_2D_3$, and even lesser amounts to the lactone.

DISCUSSION

This report presents evidence that 23(*S*),25-$(OH)_2D_3$ is an intermediate in the biosynthesis of 25-OH-D_3-26,23-lactone. The 23,25-$(OH)_2D_3$ is a major product of 25-OH-D_3 metabolism by homogenates of kidney from chickens given vitamin D (Table 2; ref. 6). It is, in fact, much more prominent than 25(*S*),26-$(OH)_2D_3$. Furthermore, 23(*S*),25-$(OH)_2D_3$ is the best substrate for the production of the lactone by these kidney preparations (Table 1). This is specific for the 23(*S*) configuration (the natural product) inasmuch as 23(*R*),25-$(OH)_2D_3$ cannot support lactone formation. In addition, both 25,26-$(OH)_2D_3$ isomers are relatively poor substrates for lactone formation with no selectivity for the naturally occurring 25(*S*),26-$(OH)_2D_3$. It would appear therefore that 23(*S*) hydroxylation precedes functionalization of C-26 for lactone formation. This contrasts with the conclusion of Hollis *et al.* (10) that 25,26-$(OH)_2D_3$ is an intermediate in lactone formation, which was based on the finding that 25,26-$(OH)_2D_3$ produced more lactone than did 25-OH-D_3 when incubated with kidney preparations. Unfortunately, 23(*S*),25-$(OH)_2D_3$ was not available to Hollis *et al.* (10) and, hence, they could not compare the relative efficiency of this vs. 25,26-$(OH)_2D_3$ in support of lactone formation. Since 25,26-$(OH)_2D_3$ is not a prominent metabolite of 25-$(OH)_2D_3$ in the lactone-producing system and since it is a relatively poor substrate for this system, it is unlikely to be a significant intermediate in lactone formation. A similar conclusion was reached by Pramanik and Napoli (11) using *in vivo* techniques.

It is unclear why both isomers of 25,26-$(OH)_2D_3$ should give a common product comigrating with the 26,23-lactone. This product is unlikely to arise from endogenous substrate, since none was seen when 23(*R*),25-$(OH)_2D_3$ was the substrate. Inversion at C-25 during incubation with one of the isomers is also not likely. It is possible that one of the products produced from one of the 25,26-$(OH)_2D_3$ isomers is not lactone. The amounts produced were too small for physical identification. Even assuming that the products of 25,26-$(OH)_2D_3$ are lactone, the data are sufficient to demonstrate that 23(*S*),25-$(OH)_2D_3$ is the likely intermediate in lactone biosynthesis.

The significance of the 25-OH-D_3-26,23-lactone pathway remains unknown. So far, both 23,25-$(OH)_2D_3$ and the lactone show no prominent vitamin D activity (unpublished results). Thus, it may represent another pathway of inactivation or it may be responsible for some previously unrecognized function of vitamin D.

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