

Studies on the Site of 1,25-Dihydroxyvitamin D₃ Synthesis *in Vivo**

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Anephric, vitamin D-deficient male rats were injected with a physiologic dose of 25-hydroxy[26,27-³H]vitamin D₃ (specific activity of 160 Ci/mmol), and 18–20 h later, intestine, bone, and serum were analyzed by high performance liquid chromatography for 1,25-dihydroxy[26,27-³H]vitamin D₃. Identical studies were carried out using sham-operated rats and rats with ligated ureters. No 1,25-dihydroxy[26,27-³H]vitamin D₃ was detected in the tissues from anephric rats, while large amounts were detected in sham-operated and ureteric ligated controls. This result demonstrates that in the nonpregnant rat, 1,25-dihydroxyvitamin D₃ is either not synthesized or is synthesized in vanishingly small amounts in bone and intestine *in vivo*, casting considerable doubt of the physiological importance of reports of *in vitro* synthesis of 1,25-dihydroxyvitamin D₃ by cells in culture derived from bone and elsewhere.

Fraser and Kodicek (1) first demonstrated that kidney is the major if not sole source of a metabolite of vitamin D that was simultaneously identified as 1,25-(OH)₂D₃¹ (2). Nephrectomy prevented biosynthesis of this compound (1), a finding clearly confirmed (3). However, at that time, the radiolabeled compounds used in those studies were of relatively low specific activity, leaving open the possibility that small amounts of 1,25-(OH)₂D₃ could be synthesized in other organs and thus not detected. Recently, cultured cells from bone (4, 5) and other tissues (6) have been reported to synthesize 1,25-(OH)₂D₃. These interesting findings therefore have challenged the exclusivity of kidney as a source of 1,25-(OH)₂D₃ *in vivo*. Since cultured cells can become transformed quickly and thus genes can become expressed that are not expressed *in vivo*, it is not clear that extrarenal synthesis of 1,25-(OH)₂D₃ does in fact occur *in vivo* in nonpregnant mammals. Certainly, extrarenal synthesis of 1,25-(OH)₂D₃ does occur in pregnant females (7) *in vivo*. Placental cells (8) as well as homogenates of this tissue can synthesize 1,25-(OH)₂D₃ *in vitro* (9). Additionally, extrarenal synthesis does occur ectopically in the disease sarcoidosis (10).

This study was designed to examine whether the nonpreg-

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¹ The abbreviations used are: 1,25-(OH)₂D₃, 1,25-dihydroxyvitamin D₃; 25OH[26,27-³H]D₃, 25-hydroxy[26,27-³H]vitamin D₃; HPLC, high performance liquid chromatography.

TABLE I
Maximum amount of putative 1,25-(OH)₂D₃ found in tissues at 18–20 h

	Nephrectomized	Ureter-ligated	Intact
Blood	ND ^a	38,000 cpm 360 fmol/ml	106,000 cpm 1,000 fmol/ml
Bone	ND	11,900 cpm 112 fmol/g	24,600 cpm 234 fmol/g
Intestine	ND	32,000 cpm 303 fmol/g	76,600 cpm 725 fmol/g

^a ND means no radioactivity was recovered above background in the 1,25-(OH)₂D₃ fraction on repeated high performance liquid chromatography columns.

nant nephrectomized rat can synthesize 1,25-(OH)₂D₃ from 25OHD₃. To maximize detection, 25OH[26,27-³H]D₃ of specific activity 160 Ci/mmol was used as the *in vivo* substrate. The results clearly confirm that these animals synthesize little or no 1,25-(OH)₂D₃, casting doubt on the *in vivo* significance of the reports that cells cultured from bone and other organs can synthesize 1,25-(OH)₂D₃. Details of the experiments and most of the results are given in Miniprint. Briefly, rats were sham-operated, nephrectomized, or had their ureters ligated, and were then given 25OH[26,27-³H]D₃ (160 Ci/mmol), and 20 h later were killed, and their tissues were extracted and chromatographed through two high performance liquid chromatography procedures. The 1,25-(OH)₂[26,27-³H]D₃ was detected by counting the radioactivity in the appropriate fractions.

EXPERIMENTAL PROCEDURES²

RESULTS

The amount of 1,25-(OH)₂[³H]D₃ bound in the tissues following two high performance liquid chromatography procedures has been computed and is shown in Table I. The lower limit of detection by our methods is of the order of 0.5 fmol/g of tissue.

DISCUSSION

The present study reaffirms the previous conclusion that in the nonpregnant mammal, 1,25-(OH)₂D₃ is for all practical purposes produced in the kidney. This raises important questions of the *in vivo* significance of the finding that cells cultured from bone (4) or other tissues (5) can produce 1,25-(OH)₂D₃. The possible reasons for the cells producing 1,25-

² Portions of this paper (including "Materials and Methods," part of "Results," Figs. 1–3, and Table II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 82M-1556, cite the authors, and include a check or money order for \$2.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

(OH)₂D₃ *in vitro* are many. It is possible that the cells in culture are transformed, thus expressing genes not expressed *in vivo*. Another possibility is that conditions *in vivo* do not permit expression of the 1 α -hydroxylase gene, while those *in vitro* do. The cells surviving and thriving in culture may not be representative of the *in vivo* population. Whatever the reason, no significant evidence for extrarenal production of 1,25-(OH)₂D₃ *in vivo* was found. This is to be contrasted to the clear demonstration of extrarenal production of 1,25-(OH)₂D₃ in the case of pregnant rats (6, 17) where the *in vivo* experiments are clearly in support of the *in vitro* experiments (8, 9). Furthermore, two laboratories have shown that anephric rats do not show a bone and intestinal response to 25OHD₃ (18–20). If extrarenal production of 1,25-(OH)₂D₃ were of significance, these results could not have been obtained.

Finally, in the hands of most investigators, no 1,25-(OH)₂D₃ could be detected in anephric patients (21–23), while occasionally, some 1,25-(OH)₂D₃ has been detected (24). It should be noted that the precision and reliability of the binding assays are not such to allow confidence in detection of small amounts of 1,25-(OH)₂D₃, nor does the bone organ culture assay respond only to 1,25-(OH)₂D₃. Even if continued investigation supports these claims, it is unlikely that such extrarenal synthesis is of any quantitative significance, since osteomalacia, hypocalcemia, and high parathyroid hormone levels are found in such patients. On the other hand, it could be of interest that in a long term anephric subject having extraordinarily high parathyroid hormone level for a long period might result in some 1 α -hydroxylase appearing at extrarenal sites. This should not, however, be misconstrued as a normally occurring phenomenon.

The very interesting report of large amounts of 1,25-(OH)₂D₃ in the blood of an anephric patient having sarcoidosis does illustrate ectopic production of 1,25-(OH)₂D₃ in this disease (9). This provides an important key to this disease and should be pursued. It, however, does not address the question of normal extrarenal production of 1,25-(OH)₂D₃.

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Studies on the Site of 1,25-Dihydroxyvitamin D₃ Synthesis *in vivo*

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Materials and Methods

Weanling male albino rats (Holtzman Co., Madison, WI) were given water and a low calcium, vitamin D-deficient diet (11) for 2 weeks. They were then divided into three groups of four rats each. Group 1 were sham-operated, group 2 were nephrectomized and group 3 had their ureters ligated as previously described (3). The latter group was used to induce uremia but allow the retention of renal tissue in the circulation. The animals were then each injected intrajugularly with 1.45 x 10⁶ CPM 25-OH-[26,27-³H]D₃ dissolved in 0.05 ml of 95% ethanol. The 25-OH-[26,27-³H]D₃ (160 Ci/mmol) was synthesized as previously described (12) and was purified by HPLC on a Zorbax-SIL (Dupont Co., Wilmington, DE) column (4.6 mm x 25 cm) using 10% isopropanol/hexane as the solvent system (13). At 18-20 h after injection, the rats were killed by decapitation, blood was collected and centrifuged to yield serum. Intestines were quickly removed as were femora and tibiae of all animals.

An aliquot of serum was used to determine phosphorus by the method of Chen *et al.* (14) and the remainder from the animals in each group was combined. Standard nonradioactive 1,25-(OH)₂D₃ (Hoffmann-La Roche Co., Nutley, NJ) was added and the sample extracted as previously described (15).

The first 25 cm of small intestine immediately distal to the pyloric sphincter was slit lengthwise, washed in 0.9% saline at 0°C, diced and homogenized in 0.9% saline. Standard 1,25-(OH)₂D₃ was added to the pooled homogenates in each group and each pooled sample extracted by the Bligh and Dyer technique (16).

The femora and tibiae from the four rats in each group were cleaned of adhering tissue, split lengthwise to remove the marrow, and frozen in liquid nitrogen. They were crushed at liquid nitrogen temperature to a powder with a mortar and pestle and weighed. After addition of standard 1,25-(OH)₂D₃, the powder was extracted with methanol-chloroform (1:1). The extraction was allowed to proceed at 4°C with stirring for 2 h and completed by the Bligh and Dyer method (16).

The nine lipid extracts were then chromatographed individually on 0.7 x 14 cm Sephadex LH-20 columns (0.7 x 14 cm) using a solvent system of 65/35 chloroform/hexane. The first 11 ml of effluent was collected and stored at -20°C. This fraction contained the 25-OH-[³H]D₃.

The next 30 ml was collected as the 1,25-(OH)₂D₃ containing fractions.

Each 1,25-(OH)₂D₃ sample was then subjected to HPLC using a Zorbax-SIL column (Dupont Co., Wilmington, DE) (4.6 mm x 25 cm) with a solvent system of 10% isopropanol/hexane (13). The 1,25-(OH)₂D₃ fraction (as determined by standard 1,25-(OH)₂D₃ absorbance at 254 nm) was collected and chromatographed on a Zorbax-ODS column (4.6 mm x 25 cm) (Dupont Co.) with a solvent system of H₂O/methanol (20:80). All fractions were collected and counted.

All HPLC was performed on a Waters instrument no. APC-GPC model 204 (Waters Associates, Milford, MA) equipped with 440 UV monitor having a 254 nm fixed wavelength. Radioactivity was determined with a Packard liquid scintillation counter model 3255 (Packard Instruments, Elk Grove Village, IL). The scintillant used was purchased from Research Products Intl. Corp. (Elk Grove Village, IL).

All solvents and chemicals used were Fischer reagent grade.

The 1,25-(OH)₂D₃ in the effluent was determined from the area under 254 absorbing peak and a standard curve of area under the peak vs. 1,25-(OH)₂D₃. The percentage recovery of standard 1,25-(OH)₂D₃ during extraction and chromatography was determined to correct the 1,25-(OH)₂[³H]D₃ values for loss during sample work-up and chromatography.

Because some background radioactivity (100 CPM) was observed in the 1,25-(OH)₂D₃ region of the chromatograms of anephric rats, this material was rechromatographed on HPLC. No radioactivity could be detected that comigrates with 1,25-(OH)₂D₃.

Results

Table II illustrates serum phosphorus concentration of each rat used in the study. Normal phosphorus levels were found in all sham-operated rats while all other rats had exceedingly high values illustrating a high degree of uremia and thus successful ureteric ligation and nephrectomy.

Both sham-operated and ureteric ligated rats produced large amounts of 1,25-(OH)₂[³H]D₃ as shown by the chromatograms of blood (Fig. 1), bone (Fig. 2), and intestine (Fig. 3). On the other hand, no 1,25-(OH)₂[³H]D₃ could be detected in the same tissue from anephric rats. The small amounts of radioactivity appearing in the 1,25-(OH)₂D₃ region was not different from background and upon further rechromatography did not comigrate with 1,25-(OH)₂D₃.

TABLE II
Serum phosphorus levels in rats used for the study.

Rat	Treatment	Serum Phosphorus Conc. mg %
1	Ureter ligation	17.8
2	Ureter ligation	24.3
3	Ureter ligation	32.8
4	Ureter ligation	27.6
Average		25.6 ± 6.0
5	Nephrectomized	31.0
6	Nephrectomized	27.6
7	Nephrectomized	34.5
8	Nephrectomized	--
Average		31.0 ± 3.0
9	Sham	8.1
10	Sham	8.6
11	Sham	7.9
12	Sham	7.6
Average	Average	8.0 ± 0.4

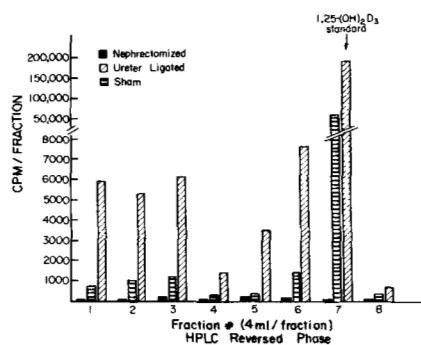


Figure 1. Radioactive profile of serum extracts after reverse-phase HPLC.

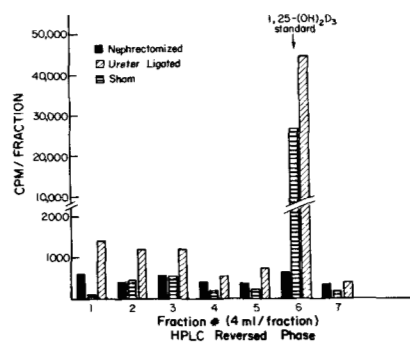


Figure 2. Reverse-phase HPLC profile of extracts from bone of rats given 25-OH-[26,27-³H]D₃.

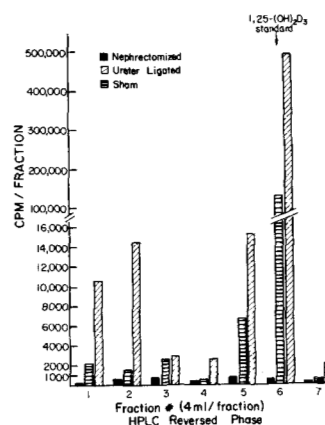


Figure 3. Reverse-phase HPLC profile of extracts from intestine from rats given 25-OH-[26,27-³H]D₃.