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Measurement of mammalian 25-hydroxyvitamin D_3 24R- and 1α-hydroxylase

(1,25-dihydroxyvitamin D_3/24,25-dihydroxyvitamin D_3/kidney/calcium)

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ABSTRACT **An *in vitro* assay of mammalian 25-hydroxyvitamin D_3 1α- and 24*R*-hydroxylases in kidney has been developed. It had been suggested that 25-hydroxyvitamin D binding protein present in mammalian blood and tissues inhibits the enzyme activities in cell-free preparations by binding the substrate 25-hydroxyvitamin D_3 more strongly than the hydroxylases bind it. This inhibitory effect is overcome by the addition of substantial amounts of unlabeled 25-hydroxyvitamin D_3 to saturate the binding sites of this protein. The resulting metabolites produced *in vitro* by rat kidney homogenates were isolated and firmly identified by ultraviolet absorption spectrometry and mass spectrometry as 1,25-dihydroxyvitamin D_3 and (24*R*)-24,25-dihydroxyvitamin D_3. Maximal 1α-hydroxylation of 25-hydroxyvitamin D_3 could be demonstrated in kidney homogenates prepared from vitamin D-deficient rats. Thyroparathyroidectomy of these rats resulted in total suppression of the 1α-hydroxylase. Homogenates of kidney from rats given vitamin D showed little or no 1α-hydroxylase and substantial 24R-hydroxylase activity. Thyroparathyroidectomy of these rats markedly increased the 24R-hydroxylase activity.**

In spite of the well-accepted fact that 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] is synthesized from 25-hydroxyvitamin D_3 (25-OH-D_3) *in vivo* by the kidney of vitamin D-deficient chickens and rats (1) and the demonstration *in vitro* of 1α-hydroxylation of 25-OH-D_3 in kidney tissue of vitamin D-deficient birds (2, 3), measurement of 1-hydroxylation by rat kidney homogenates or mitochondria has not been possible *in vitro*. A factor in rat kidney homogenate inhibits *in vitro* 1-hydroxylation of 25-OH-D_3 by vitamin D-deficient chicken kidney homogenate (4). The inhibitor has been found in many rat tissues, in blood, and in pig kidney tissue (4). Thus, it has been suggested that the failure of the rat kidney homogenate to carry out *in vitro* hydroxylation is due to the presence of the inhibitor. The inhibitory factor has been isolated from rat serum and shown to be the vitamin D transport protein with a molecular weight of 52,000 (5) and the ability to bind 25-OH-D_3 preferentially (6). It therefore is likely that this protein inhibits the 1-hydroxylase enzyme by reducing the availability of 25-OH-D_3 substrate. Although it has been suggested that addition of a large amount of 25-OH-D_3 to the incubation medium can overcome the inhibitory effect, it is difficult to use the radioactive substrate because of resulting isotope dilution unless a tremendous amount of radioactivity is used (7).

We have developed a technique for the measurement of mammalian renal 25-OH-D_3 1-hydroxylase activity that uses substantial amounts of nonradioactive 25-OH-D_3 substrate to saturate the inhibitor protein and high-performance liquid chromatography (HPLC) for separation, identification, and quantitation of the dihydroxy metabolites produced *in vitro*. Although the technique was originally developed for 25-OH-D_3 1-hydroxylase assay, it can be applied to measurement of the 25-OH-D_3 24-hydroxylase as well. This method now makes possible a study of regulation of the renal 25-OH-D_3 hydroxylases in the rat. With this method it can be shown clearly that thyroparathyroidectomy will suppress the 1α-hydroxylase and stimulate the 24R-hydroxylase.

MATERIALS

Vitamin D Compounds. The vitamin D_3 was purchased from Philips Roxane (New York, NY). The 25-OH-D_3 was a gift from Upjohn and (24*R*)-24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ were gifts from Hoffmann–LaRoche. The 1,25-$(OH)_2$[26,27-^{3}H]D_3 (specific activity, 160 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was synthesized by Napoli *et al.* (8). The 24,25-$(OH)_2$ [3α-^{3}H]D_3 was synthesized biologically by the method of Tanaka *et al.* (9) from 25-OH-[3α-^{3}H]D_3 which was synthesized by the method of Yamada *et al.* (10).

Animals. Weanling male rats were purchased from Holtzman (Madison, WI) and fed a vitamin D-deficient diet (11) for 6 weeks. They were kept in overhanging wire cages with the diet and water fed *ad lib.* Vitamin D-supplemented rats were given 25 international units of vitamin D_3 in 0.1 ml of cottonseed/soy bean oil (Wesson) daily by mouth.

Incubation of Kidney Homogenate. Rats were killed by decapitation, and the kidneys were removed and placed in ice-cold 15 mM Tris acetate buffer, pH 7.4/0.19 M sucrose/2 mM magnesium acetate. A 5% (wt/vol) homogenate of kidney was prepared in the buffer, and the incubation was carried out in a 125-ml erlenmeyer flask containing 100 mg of kidney tissue, 15 mM Tris acetate, 0.19 M sucrose, 2 mM magnesium acetate, and 25 mM sodium succinate in a final volume of 3 ml. One hundred micrograms of 25-OH-D_3 dissolved in 25 μl of 95% ethanol was added to each flask. The mixtures were incubated at 37°C at 100 oscillations/min for 20 min unless otherwise indicated. The reaction was stopped by addition of 20 ml methanol/chloroform, 2:1 (vol/vol). Tracers 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ (5000 cpm each) were dissolved in 50 μl of 95% ethanol and added to the assay flask when indicated to monitor recoveries of the products through extraction and chromatography. Extraction was carried out as described by Lund and DeLuca (12).

Sephadex LH-20 Chromatography of the Extract. The lipid extract was evaporated to dryness under reduced pressure, and the residue was dissolved in chloroform/hexane, 65:35 (vol/vol), and applied to a Sephadex LH-20 column (0.7 × 14 cm) packed in the same solvent mixture as described by Holick and DeLuca (13). The column was eluted with 36 ml of the same solvent. The last 25 ml of effluent, which contained the dihydroxy

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Abbreviations: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 25-OH-D_3, 25-hydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; HPLC, high-performance liquid chromatography.

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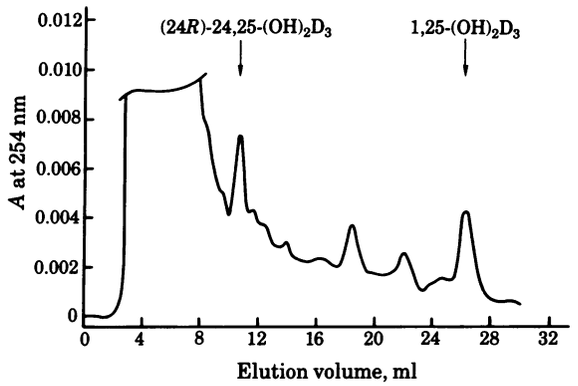

FIG. 1. HPLC of 25-OH-D_3 products generated by rat kidney homogenates. HPLC using a Zorbax SIL column (4.6 mm × 25 cm) was eluted with 12% 2-propanol in hexane at 1000 psi and a flow rate of 2 ml/min. Arrows show the elution volume of authentic (24*R*)-24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$. Fractions between 9 and 12 ml were collected and subjected to the reversed-phase HPLC shown in Fig. 2*A*; fractions between 25 and 28 ml were collected and subjected to the reversed-phase HPLC shown in Fig. 2*B*.

metabolites, was collected; the first 11 ml, which contained unchanged substrate and yellow lipid, was discarded (or could be used to recover unused substrate). The column had been previously calibrated with radioactive 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$.

HPLC for Enzyme Assay. Authentic 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ were injected into a Waters model ALC/GPC 204 HPLC (Waters Associates) using a Zorbax SIL column (DuPont) (4.6 mm × 25 cm), to establish elution volumes for each compound. Straight phase HPLC was performed at 1000 psi (700 kPa) and a flow rate of 2 ml/min with a solvent of 12% 2-propanol in hexane. The lipid extract purified through a Sephadex LH-20 column was dissolved in 200 μl of the same solvent and injected into the HPLC. A representative HPLC profile is shown in Fig. 1. The fractions containing 24,25-$(OH)_2D_3$ (9–12 ml) and 1,25-$(OH)_2D_3$ (25–28 ml) were collected separately for further purification and quantitation of the metabolites by reversed phase HPLC.

The reversed phase HPLC using Lichrosorb RP-18 (4.6 mm × 25 cm) (E. Merk, Darmstadt, Federal Republic of Germany) was performed with 22% H_2O in methanol for 24,25-$(OH)_2D_3$ and 20% H_2O in methanol for 1,25-$(OH)_2D_3$. This column was developed at 1300 psi and a flow rate of 2 ml/min. The column had been calibrated with authentic (24*R*)-24,25-$(OH)_2D_3$ or with 1,25-$(OH)_2D_3$ (Fig. 2). Each fraction (0.8 ml) around the 24,25-$(OH)_2D_3$ area or 1,25-$(OH)_2D_3$ area was collected, dried, and assayed to calculate recovery of 24,25-$(OH)_2[^3H]D_3$ or 1,25-$(OH)_2[^3H]D_3$. At the same time, the chromatographic profiles of the metabolites produced *in vitro* were obtained by ultraviolet detection at 254 nm.

Standard curves were made by plotting the area under the peak obtained by the ultraviolet detector against the amount of authentic (24*R*)-24,25-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$ injected. Peak areas of 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ were measured and the metabolites produced *in vitro* were calculated from the standard curves. The metabolite concentration was further corrected by dividing by the fraction of radioactive metabolite recovered. Recovery of the labeled internal standards was routinely 80 ± 5% (mean ± SD). When routine assays are performed, radioactive internal standards need not be applied once assay recoveries are accurately known and are reproducible.

Preparation of Metabolites for Identification. Kidneys from vitamin D-deficient rats and from vitamin D-supplemented rats

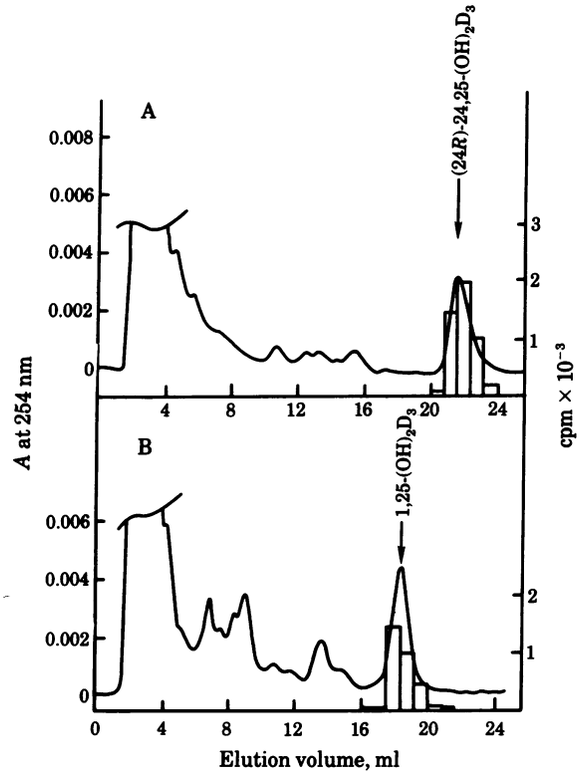

FIG. 2. (*A*) Reversed-phase HPLC of 24,25-$(OH)_2D_3$ collected from straight-phase HPLC (Fig. 1). HPLC used Lichrosorb RP-18 column (4.6 mm × 25 cm) and elution with 22% H_2O in methanol at 1300 psi and flow rate of 2 ml/min. Elution position of authentic (24*R*)-24,25-$(OH)_2D_3$ is shown. Each 0.8-ml fraction between 15 and 24 ml was collected; the radioactivity was measured (bars), the area under the peak 24,25-$(OH)_2D_3$ obtained by ultraviolet detector at 254 nm was measured, and the amount of the compound was calculated with a standard curve. (*B*) Reversed-phase HPLC of 1,25-$(OH)_2D_3$ collected from straight-phase HPLC (Fig. 1). HPLC used a Lichrosorb RP-18 column (4.6 mm × 25 cm) and elution with 20% H_2O in methanol at 1300 psi and flow rate of 2 ml/min. Elution position of authentic 1,25-$(OH)_2D_3$ is shown. Each 0.8-ml fraction was assayed for radioactivity (bars) and the amount of 1,25-$(OH)_2D_3$ detected by ultraviolet monitor was measured.

were combined, and a 5% homogenate of the kidney tissue was prepared in ice-cold 15 mM Tris acetate, pH 7.4/0.19 M sucrose/2 mM magnesium acetate. Incubation conditions were as described above except for the incubation time. Five mixtures were simultaneously prepared and incubated for 1 hr. They were combined, extracted, and purified through Sephadex LH-20; the chromatographic profile of the straight-phase HPLC was similar to that in Fig. 1 except that the peaks were larger. The fractions containing the putative 24,25-$(OH)_2D_3$ were collected, dried, dissolved in 22% H_2O in methanol, and injected for HPLC with a Lichrosorb RP-18 column as used in Fig. 2*A*. The compound was recycled twice and collected. The fractions containing 1,25-$(OH)_2D_3$ were treated in the same manner except that reversed-phase HPLC was performed with 20% H_2O in methanol as in Fig. 2*B*. The putative 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ then were purified once more through straight-phase HPLC as in Fig. 1. Recycling of putative compounds by an additional straight-phase HPLC was required for the elimination of impurities that interfere with mass spectrometry. About 1 μg each of purified 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ was obtained for physical measurements.

Physical Methods. Ultraviolet absorption spectra were recorded with a Beckman 24 recording spectrophotometer. Determination of radioactivity was carried out in toluene counting

solution (14) with a Packard liquid scintillation counter (model 3255). Mass spectrometry was carried out with an A.E.I. MS 9 mass spectrometer.

RESULTS

A typical HPLC profile of lipid extract of an incubation mixture is shown in Fig. 1. Although the extract was prepurified through a Sephadex LH-20 column, many peaks of ultraviolet absorbance were detected. The heterogeneous peaks from Fig. 1 containing 24,25-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$ could be easily resolved on reversed-phase HPLC (Fig. 2).

Both purified compounds showed typical vitamin D ultraviolet absorption maximum at 265 nm and a minimum at 228 nm as determined in 95% ethanol indicating an intact 5,6-*cis*-triene system. Mass spectra of *in vitro* produced metabolites were identical to those of their respective standards. The mass spectrum of 1,25-$(OH)_2D_3$ produced *in vitro* showed a molecular ion of 416 and fragments of *m/e* 398 ($M^+ - H_2O$), 380 ($M^+ - 2H_2O$), 152 (ring A + C − 6 + C − 7), and 134 (152 − H_2O). The fragments *m/e* 134 and 152 indicated that the added oxygen is on the A ring because they were increased by 16 above the same fragment arising from 25-OH-D_3. The mass spectrum of 24,25-$(OH)_2D_3$ produced *in vitro* showed a molecular ion of 416 with fragments *m/e* 118 and 136, indicating that the additional oxygen is not on the A ring. Furthermore, fragments 271 (M^+ − side chain) and 253 (271 − H_2O) were identical to those of vitamin D_3 and 25-OH-D_3, indicating that the additional oxygen function is in the side chain. The relative intensities identical to those of the standard, together with ultraviolet absorption spectrum and chromatographic character, confirm that this *in vitro* produced compound is 24,25-$(OH)_2D_3$.

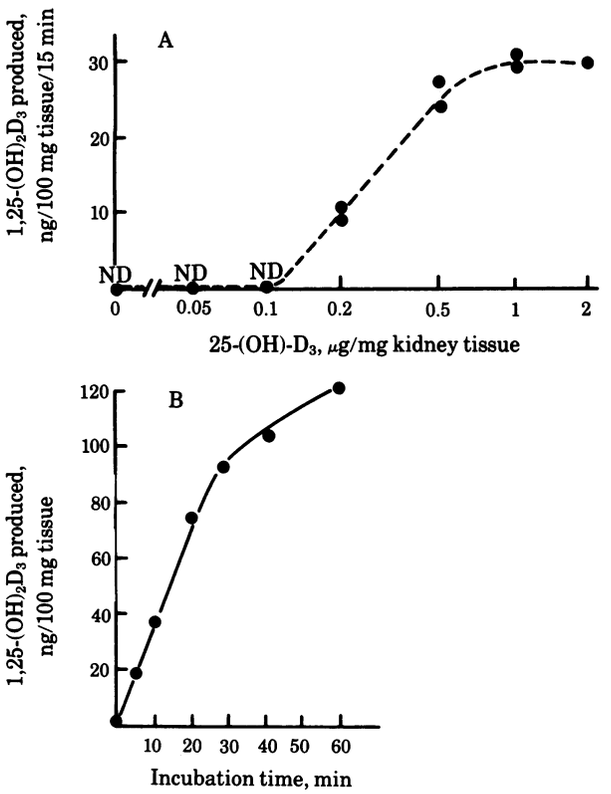

FIG. 3. (*A*) Dependency of *in vitro* production of 1,25-$(OH)_2D_3$ by rat kidney homogenates on the concentration of 25-OH-D_3. The incubation was carried out as described in the text with 0, 5, 10, 20, 50, 100, or 200 μg of 25-OH-D_3 dissolved in 50 μl of 95% ethanol. Incubation time was 15 min. ND, not detectable (<5 ng). (*B*) Time course of *in vitro* production of 1,25-$(OH)_2D_3$ by rat kidney homogenate. Incubation conditions were as for *A* except that amount of 25-OH-D_3 was 100 μg/100 mg of tissue and incubation time was varied.

Table 1. Influence of vitamin D administration and thyroparathyroidectomy on 25-OH-D_3 24R- and 1-hydroxylase activities in rat kidneys

Operation*	Rats no.	1,25-$(OH)_2D_3$ produced, ng/100 mg tissue/ 20 min†	24,25-$(OH)_2D_3$ produced, ng/100 mg tissue/ 20 min†
Sham	8	80.7 ± 2.4	ND
8 hr post-TPTX	3	43.8 ± 4.0	ND
18 hr post-TPTX	6	ND	ND
	Vitamin D-replete		
Sham	5	<6	44.9 ± 5.0
8 hr post-TPTX	4	ND	108.3 ± 10.9

Rats had been fed the vitamin D-deficient diet for 6 weeks. The vitamin D-replete rats were prepared as described in the text.
* TPTX, thyroparathyroidectomy.
† Shown as mean ± SD. ND, not detectable (<5 ng).

Although the rat renal 25-OH-D_3-hydroxylases are also found in the mitochondrial fraction (unpublished data), the assay method has been developed for whole homogenate. The substrate required to overcome the effect of the inhibitor protein was first determined. A 5% homogenate of kidney from rats that had been fed the vitamin D-deficient diet for 6 weeks was incubated with various concentrations of 25-OH-D_3. Dependency of *in vitro* 1-hydroxylation of 25-OH-D_3 by rat kidney homogenate on amount of substrate is shown in Fig. 3*A*. At least 1 μg of 25-OH-D_3 per mg of kidney tissue (wet weight) was necessary for maximal reaction rate. With this substrate concentration, production of 1,25-$(OH)_2D_3$ was linear for up to 30 min (Fig. 3*B*). Therefore, for 100 mg of kidney tissue, a 20-min incubation time with 100 μg of 25-OH-D_3 was selected for routine assay of 1α-hydroxylase.

The assay method was tested for reproducibility by using a single homogenate of pooled kidneys from vitamin D-deficient rats from which samples were taken for assay in separate incubation vessels. The 1-hydroxylase activities from four individual assays showed a mean (±SD) of 88.0 ± 4.0 ng of 1,25-$(OH)_2D_3$ produced per 100 mg of tissue in 20 min. When samples from individual rats of the same age and on the same diet for the same length of time were assayed, the mean (±SD) was 83.3 ± 8.4 ng of 1,25-$(OH)_2D_3$ produced per 100 mg tissue in 20 min (five rats).

In a series of experiments, modulation of the renal hydroxylases by vitamin D and the parathyroid glands was assessed. Vitamin D deficiency markedly increased the 1-hydroxylase activity and suppressed the 24-hydroxylase activity; vitamin D supplementation suppressed 1α-hydroxylase and stimulated 24R-hydroxylase (Table 1). Thyroparathyroidectomy of vitamin D-deficient rats suppressed the 1-hydroxylase activity to half by 8 hr after operation and to a nondetectable level by 18 hr after operation. No detectable 24,25-$(OH)_2D_3$ was produced by preparations from vitamin D-deficient rats. Thyroparathyroidectomy of vitamin D-fed rats increased the 24-hydroxylase activity.

DISCUSSION

Demonstration of *in vitro* production of 1,25-$(OH)_2D_3$ by homogenate of mammalian kidney tissue has been largely unsuccessful in the past except for a few cases. Midgett *et al.* (15) and Henry and Norman (16) reported that kidney of mammals such

as dog, man, and rat can carry out the 1-hydroxylation of radioactive 25-OH-D_3 *in vitro* but the amounts of 1,25-$(OH)_2D_3$ were far below the level produced by chicken kidney preparations or by mammalian kidney *in vivo* (17, 18). Furthermore, these results in the rat could not be reproduced and identification of the products was not convincing (16).

More recently, Saarem *et al.* (19) reported the formation of 1,25-$(OH)_2[^3H]D_3$ from 25-OH-$[^3H]D_3$ by incubation of isolated mitochondria from the kidneys of rachitic pigs, and Sunaga *et al.* (20) reported the *in vitro* 24- and 1-hydroxylation of 25-OH-$[^3H]D_3$ by *in vitro* incubation of kidney homogenate from New Zealand White rabbits. The level of activity reported appeared to be low and could have been affected by the presence of inhibitor protein (4). Although the physiological significance of the inhibitor protein is unknown, the presence of the protein in variable quantities could complicate measurements and interpretation.

We have now successfully demonstrated the *in vitro* production of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ by rat kidney homogenates in amounts sufficient for detection by HPLC. The metabolites were firmly identified by mass spectrometry and ultraviolet absorption spectrophotometry. The assay method developed here can be applied to measurement of the renal hydroxylases of other mammalian species as well as to the study of regulation of renal 25-OH-D_3 hydroxylases by using rats or other mammalian species.

In the assay method, prepurification of the extract of the reaction mixture by a batch column was found to be advantageous not only for the removal of lipid but also for removal of ultraviolet absorbing substances more polar than 1,25-$(OH)_2D_3$. Furthermore, a sample purified through Sephadex LH-20 column chromatography can be injected into HPLC repeatedly without need for washing of the HPLC column between samples. In addition, 20 batch Sephadex columns can be run simultaneously, shortening the overall time needed for chromatography and, hence, assay.

It was found that both straight- and reversed-phase HPLC are necessary for baseline separation of the metabolites and for accurate quantitation of the products. Separation of the metabolites with only reversed-phase HPLC without previous straight-phase HPLC did not give adequate resolution of the metabolites, and the presence of several ultraviolet absorbing substances eluting after the desired metabolite delays injection of the next sample.

It is surprising that use of 1 μg of 25-OH-D_3 per mg of tissue is necessary to overcome the inhibitory effect of the binding protein and saturate the 1α-hydroxylase of rat kidney. As shown in Fig. 3*B*, the *in vitro* production of 1,25-$(OH)_2D_3$ is linear for up to 30 min at this substrate concentration. Carrying out the reaction at initial reaction velocity at substrate saturation provides the most sensitive and reliable hydroxylase assays.

Other important advantages of the assay method reported here are that it is rapid, does not involve the routine use of expensive radiochemicals, and reduces chromatographic costs. Unused substrate can also be collected from the Sephadex column, purified and reused; the quantities of products produced permit the ready use of physical methods such as mass spectrometry to identify them.

By using this method it has been shown that vitamin D-deficient rat kidney has high 25-OH-D_3 1α-hydroxylase activity, comparable to that in rachitic chick homogenate (42 pg/mg tissue per min vs. 40 pg/mg per min). Vitamin D administration suppresses 1α-hydroxylase activity and induces 24R-hydroxylase. Thyroparathyroidectomy completely suppresses 1α-hydroxylase and increases 24R-hydroxylase. These results, previously suspected (21), have now been established firmly by the present methods.

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