Metabolism of [3α-3H] 25-Hydroxyvitamin D3 in Kidneys Isolated from Normal and Vitamin D2-intoxicated Rats

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Summary With the availability of A-ring labelled 25OHD2, [3α-3H] 25OHD2, we have performed the present study to examine the metabolism of 25OHD2 using physiological substrate concentrations in perfused kidneys isolated from both normal and vitamin D2-intoxicated rats. Our results indicate that [3α-3H] 25OHD2 is metabolized into both 24(S),25,28-trihydroxyvitamin D3 [24(S),25,28(OH)3D3] and 24(R),25,26-trihydroxyvitamin D3 [24(R), 25,26(OH)3D3], and the amounts of these two metabolites produced in the kidney of vitamin D2-intoxicated rat were about 3–5 times higher than those produced in the kidney of normal rat. Similar results were also obtained with rat kidney homogenates incubated with [3α-3H] 25OHD2. Furthermore, we noted that the production of both 24(S),25,28(OH)3D3 and 24(R),25,26(OH)3D3 in the kidney homogenates of vitamin D2-intoxicated rats increased with the time of incubation and then subsequently decreased. The decrease in both 24(S),25,28(OH)3D3 and 24(R),25,26(OH)3D3 coincided with an increase in the fraction of total radioactivity distributed in the aqueous phase of the kidney homogenates. This finding suggested the possibility of further metabolism of 24(S),25,28(OH)3D3 and 24(R), 25,26(OH)3D3 into polar water-soluble metabolite(s). We then measured the radioactivity in the aqueous phase of kidney homogenates of both normal and vitamin D2-intoxicated rats incubated with [3α-3H] 25OHD2. It was noted that the amount of radioactivity in the aqueous phase of kidney homogenates of normal rat is lower than that present in the aqueous phase of kidney homogenates of normal rats. Thus, our study provides evidence for the first time for the formation of both 24(S),25,28(OH)3D3 and 24(R),25,26(OH)3D3 under physiological conditions, and the possibility of their further metabolism into as yet unidentified polar water-soluble metabolite(s). As the formation of all these metabolites is increased in the kidney of vitamin D2-intoxicated rats when compared to normal rats, it appears that the increased rate of metabolism of 25OHD2 during hypervitaminosis D2 plays a significant role in the deactivation of 25OHD2.

Key Words polar water-soluble metabolite(s), hydroxylation, kidney, metabolism, vitamin D2-intoxicated rats, 25OHD2

In previous studies, we demonstrated that the side chain metabolic pathways of vitamin D3 compounds differ from those of vitamin D2 compounds.

Abbreviations: 25OHD3, 25-hydroxyvitamin D3; [1α,25(OH)2D3], 1α,25-dihydroxyvitamin D3; [1α,25(OH)2D2], 1α,25-dihydroxyvitamin D2

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is important to understand the inactivation pathways of 25OHD₂. Previously we reported that 25OHD₂ is metabolized into 24(R),25-dihydroxyvitamin D₃ [24(R), 25(OH)₂D₃], 24(S),25,28-trihydroxyvitamin D₃ [24(S), 25,28(OH)₂D₃] and 24(R),25,26-trihydroxyvitamin D₂ [24(R),25,26(OH)₂D₂] in the isolated kidneys of vitamin D₂-intoxicated rats perfused with pharmacological concentrations of 25OHD₂ (1). Furthermore, using radio-labelled vitamin D₂, we also indicated that both 24(S),25,28(OH)₂D₃ and 24(R),25,26(OH)₂D₂ circulate in a vitamin D₂-intoxicated rat in significant amounts. The amount of C-28 hydroxylated metabolite was more than that of the C-26 hydroxylated metabolite (1). These studies indicated that C-28 hydroxylation plays an important role in the metabolism of 25OHD₂ under pharmacological conditions. However, until the present time, the formation of 24(S),25,28(OH)₂D₃ and 24(R),25,26(OH)₂D₂ in normal rats using physiological substrate concentrations has not been studied. Therefore, the present study was performed with the following aim: to study the metabolism of 25OHD₂ into 24(S),25,28(OH)₂D₃ and 24(R),25,26(OH)₂D₂ in normal and vitamin D₂-intoxicated rats using physiological substrate concentrations of 25OHD₂.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (about 350 g) were purchased from Zivic-Miller Laboratories, Inc., Allison Park, PA, and placed in individual hanging wire cages. We induced hypervitaminosis D₂ into these rats by administering 1,000 nmol of vitamin D₂ (oral dose in 0.1 mL of Wesson oil) each day for a period of 14 d. The last dose of vitamin D₂ was administered to the rats 24 h prior to the isolation of the kidney. The serum calcium concentrations of the control rats were determined on the day of sacrifice. As expected, the vitamin D₂-intoxicated rats were hypercalcemic, and their serum calcium levels were in the range of 13–14 mg/100 mL. The serum calcium concentrations of the normal rats were in the range of 10–11 mg/100 mL.

Vitamin D compounds. Vitamin D₂ was purchased from Sigma Chemical Co. (St. Louis, MO). 25OHD₂ was a gift from Drs. J. A. Campbell and J. Babcock (Upjohn Co., Kalamazoo, MI). 24(R),25(OH)₂D₂, 24(S),25,28(OH)₂D₂ and 24(R),25,26(OH)₂D₂ were produced from 25OHD₂ in the isolated perfused rat kidney as described before (1). 1α,25(OH)₂D₃ and 25,28(OH)₂D₂ were synthesized at Hoffmann-La Roche, Inc., Nutley, NJ. [3α-³H] vitamin D₂ was synthesized as described earlier (6).

High-Performance Liquid Chromatography (HPLC). Ultraviolet (UV) absorbance spectra were taken in 2-propanol with a Beckman DU8 recording spectrophotometer. HPLC was performed with a Waters Model 600 equipped with a detector (Model 440) to monitor UV-absorbing material at 254 nm (Waters Associates, Milford, MA). All HPLC solvents were from Burdick and Jackson Laboratories, Muskegan, MI.

Lipid extraction. Lipid extraction of the perfusate was performed according to the procedure of Bligh and Dyer (7), except that methylene chloride was substituted for chloroform.

Synthesis of [3α-³H] 25OHD₂ using the technique of liver perfusion. [3α-³H] 25OHD₂ was biologically synthesized from [3α-³H] vitamin D₂ using a liver perfusion system. Liver perfusion was performed by the isolation of livers from vitamin D₂-deficient rats. The vitamin D₂-deficient rats were anesthetized with nembutal, and the portal vein and bile duct were cannulated. The liver was isolated from the animal and perfused with 200 mL of perfusate containing 5 μCi of [3α-³H] vitamin D₂ for a period of 4 h as described earlier (8). A total of four liver perfusions were performed.

The final perfusate of all four liver perfusions was pooled to obtain a total perfusate of about 800 mL, which was subjected to the lipid extraction procedure. The final lipid extract was divided into 10 portions by dissolving in 1 mL of n-hexane-2-propanol (97 : 3). Each 100 μL portion was then subjected to a Zorax-SII, column (25 cm x 4.6 mm) eluted with hexane-2-propanol (97 : 3) at a flow rate of 2 mL/min. From the first HPLC system, we obtained a clear separation of 25OHD₂ from vitamin D₂. The radioactive material in the region of 25OHD₂ from the first HPLC system was then passed through two additional HPLC systems as described below. The radioactive material eluting at 12–18 mL (the elution volume of standard 25OHD₂) from all 10 HPLC runs was pooled and dried under nitrogen. The residue was divided into four portions by dissolving in 400 μL of methylene chloride-2-propanol (98 : 1.5). Each 100 μL portion was then subjected to a second HPLC using methylene chloride-2-propanol (98 : 1.5) as the solvent system. The radioactive material eluting at 12–16 mL (the elution volume of standard 25OHD₂) from all 4 HPLC runs was pooled and dried under nitrogen. The final purified material was then dissolved in 100 μL of n-hexane-2-propanol (97 : 3) and subjected to the first HPLC run. The radioactive material eluting at 12–18 mL was collected and dried under nitrogen. About 1 μCi of [3α-³H] 25OHD₂ was obtained for each 5 μCi of [3α-³H] vitamin D₂ used in a single liver perfusion experiment. The final purified radioactive material was dissolved in 5 mL of ethanol and stored for kidney perfusion studies.

Study of [3α-³H] 25OHD₂ metabolism in the kidney of normal and vitamin D₂-intoxicated rats using the technique

Fig. 1. Chemical structures of vitamins D₂ and D₃.
of kidney perfusion. This experiment was designed to study the renal metabolism of \([3\alpha^-3H] 25OHD_2\). Perfusions of kidneys isolated from both normal and vitamin D2-intoxicated rats were performed as described before in detail (9). The metabolism of 25OHD_2 at a substrate concentration of \(3 \times 10^{-10} \text{ M}\) was studied by introducing 1 µCi of 25OHD_2 into 100 mL of perfusate after a 5-min stabilization period following isolation of the kidney. Aliquots of perfusate (2 mL) were taken out of the perfusion circuit every 1 h, and the kidney perfusion was continued for 4 h. The various radio-labelled metabolites of 25OHD_2 and remaining unmetabolized \([3\alpha^-3H] 25OHD_2\) in the lipid extract of perfusate samples were analyzed by HPLC as described later. In this study, we also performed a control perfusion experiment in the absence of a kidney and demonstrated that there was no metabolism of \([3\alpha^-3H] 25OHD_2\) in the perfusion apparatus in the absence of a kidney (data not shown).

Study of \([3\alpha^-3H] 25OHD_2\) metabolism into lipid-soluble metabolites in the kidney homogenates of normal and vitamin D2-intoxicated rats. The kidney homogenates were prepared as described earlier (10). Briefly, the kidneys of normal and vitamin D2-intoxicated rats were removed and placed in ice-cold Tris acetate buffer (pH 7.4) (15 mM) containing sucrose (0.19 M), magnesium acetate (2 mM) and sodium succinate (25 mM). The kidney homogenates (10% w/v) were prepared in Tris-acetate buffer. The metabolism of 25OHD_2 at a substrate concentration of \(3 \times 10^{-10} \text{ M}\) was studied by adding 0.3 µCi of \([3\alpha^-3H] 25OHD_2\) to 30 mL of the homogenate. Oxygen gas was flushed for 1 min into each flask on ice, and the homogenates were incubated at 37°C for 1 h. The reaction was stopped by adding 30 mL of methanol. Aliquots of homogenate (5 mL) were taken out at various time intervals up to an incubation period of 1 h and were subjected to lipid extraction as described before. The various radio-labelled metabolites of 25OHD_2 and the remaining unmetabolized 25OHD_2 in the lipid extracts of homogenate samples were analyzed by HPLC. In this study, we also performed a control experiment using kidney homogenates preheated at 100°C for 5 min and demonstrated that there was no metabolism of \([3\alpha^-3H] 25OHD_2\) (data not shown).

Analysis of lipid extracts of kidney perfusate and homogenate samples for various metabolites of \([3\alpha^-3H] 25OHD_2\) by HPLC. Lipid extracts of perfusate (2 mL each) or homogenate samples (5 mL each) obtained at different time intervals from the above kidney perfusion and kidney homogenate experiments were analyzed by HPLC. A clear separation of all the metabolites of 25OHD_2 was obtained by a single HPLC run under the chromatographic conditions described in detail in the legend for Fig. 2. The amounts of unmetabolized 25OHD_2 and each metabolite present in the lipid extract of each 2 mL perfusate and 5 mL homogenate were quantified by measuring the radioactivity. Non-radioactive 25,28(OH)_2D_2 and 1α,25(OH)_2D_2 (0.5 µg each) were added as internal standards to the perfusate and homogenate samples before lipid extraction to monitor the recovery of vitamin D_2 metabolites, and thus appropriate corrections were made for the losses that might have occurred during the procedures of lipid extraction and HPLC.

Study of \([3\alpha^-3H] 25OHD_2\) metabolism into water-soluble metabolites in the kidney homogenates of normal and vitamin D2-intoxicated rats. The kidney homogenates of either normal or vitamin D_2-intoxicated rats \((n=4)\) were prepared as described earlier. The metabolism of 25OHD_2 at a substrate concentration of \(3 \times 10^{-10} \text{ M}\) was studied by adding 30,000 cpm of \([3\alpha^-3H] 25OHD_2\) to 3 mL of homogenate and incubated for 1 h. The incubation conditions are as described earlier. At the end of 1 h, the incubations were stopped by adding 3 mL of methanol. Lipid extraction of the homogenate samples was performed as described earlier. The methylene chloride layer and water-methanol layers of the homogenate samples were separated and the total radioactive counts distributed in each layer were estimated by measuring radioactivity in 1/10 of the total volume of each layer.

Statistical analysis. Values were calculated as mean±standard error (SE). Significance levels were determined by student’s t-test.

RESULTS

Metabolism of \([3\alpha^-3H] 25OHD_2\) by the perfused kidney isolated from normal and vitamin D_2-intoxicated rats

Figure 2 shows a comparison of the radioactive HPLC profiles of the lipid extracts obtained from 2 mL of perfusate after perfusing the kidney of normal or vitamin D_2-intoxicated rats with \([3\alpha^-3H] 25OHD_2\) for 4 h. It can be seen from Fig. 2 that 25OHD_2 was metabolized in perfused kidneys isolated from both normal (Panel A) and vitamin D_2-intoxicated rats (Panel B) into three metabolites \([24(R),25(OH)_2D_2; 24(S),25,28(OH)_3D_2; 24(R),25,26(OH)_3D_2]\). The amount of unmetabolized substrate \((25OHD_2)\) at the end of 4 h was about 1.8 times higher in the kidney perfusate of normal rat when compared to the vitamin D_2-intoxicated rat. Furthermore, the amounts of trihydroxylated metabolites, \([24(S),25,28(OH)_3D_2; 24(R),25,26(OH)_3D_2]\), produced in the kidney of vitamin D_2-intoxicated rat were about 4 times higher when compared to the corresponding metabolites produced in the kidney of normal rats. Out of the two trihydroxylated metabolites, we also noted that the amount of \([24(S),25,28(OH)_3D_2]\) was about 3 times higher when compared to \([24(R),25,26(OH)_3D_2]\) in the kidney perfusates of both normal and vitamin D_2-intoxicated rats. However, there was no direct conversion of 25OHD_2 into \([25,28(OH)_3D_2]\).

The rate of disappearance of 25OHD_2 from the perfusate and the rate of appearance of \([24(R),25(OH)_2D_2; 24(S),25,28(OH)_3D_2; 24(S),25,28(OH)_3D_2; 24(R),25,26(OH)_3D_2]\) into the perfusate are shown in Fig. 3. The amount of substrate reduction in the kidney perfusates of both the normal and vitamin D_2-intoxicated rats with perfusion time (Panel A) is obvious. However, at every time interval, the amount of unmetabolized substrate in the kidney perfusate of the vitamin D_2-intoxicated rat was around...
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Fig. 2. HPLC profiles of lipid soluble metabolites of [3$\alpha$-$^3$H] 25OHD$_2$, produced in an isolated perfused rat kidney. Panel A: normal rat; Panel B: vitamin D$_2$-intoxicated rat. A kidney perfusion was performed with 1 $\mu$Ci of [3$\alpha$-$^3$H] 25OHD$_2$ in 100 mL of perfusate for a period of 4 h. Lipid extracts of perfusate samples (2 mL each) were analyzed by HPLC under the following chromatographic conditions: Zorbax-SIL column, 25 cm$\times$4.6 mm; flow rate, 2 mL/min. The column was first eluted with hexane-2-propanol (97.5 : 2.5) until 25, 28(OH)$_2$D$_2$ was eluted out of the column. Then the first solvent system was changed to a second solvent system (hexane-2-propanol, 95 : 5), keeping the flow rate the same to elute more polar metabolites of vitamin D$_2$ out of the column. Fractions of 2 mL were collected and the HPLC effluent in each fraction was divided into two equal portions. The first portion was used to measure the radioactivity, which is depicted in the figure. The second portion was used for rechromatography of each major metabolite of 25OHD$_2$ on a second HPLC run using a methylene chloride-2-propanol (94 : 6) mixture as the solvent system. Arrows indicate the elution positions of the various authentic standards of 25OHD$_2$ metabolites. Unlabelled 25, 28(OH)$_2$D$_2$ and 1$\alpha$, 25(OH)$_2$D$_2$ (0.5 $\mu$g each) were added to each 2 mL perfusate sample at the time of lipid extraction to monitor recovery of radiolabelled 25OHD$_2$ metabolites. The recovery of unlabelled 25, 28(OH)$_2$D$_2$ and 1$\alpha$,25(OH)$_2$D$_2$ in each perfusate sample was determined by its UV absorbance at 254 nm and was found to be approximately 90%. In both the HPLC systems, the radiolabelled 25OHD$_2$ metabolites co-migrated with the standard cold 25OHD$_2$ metabolites.

1.5 times lower than that in the kidney perfusate of normal rat. Further the amounts of 24(R),25(OH)$_2$D$_2$ (Panel B), 24(S),25,28(OH)$_3$D$_2$ (Panel C) and 24(R),25, 26(OH)$_3$D$_2$ (Panel D) increased with perfusion time in both normal and vitamin D$_2$-intoxicated rats. It was noted that, at all the time intervals studied, the amounts of 24(S),25,28(OH)$_3$D$_2$ and 24(R),25, 26(OH)$_3$D$_2$ produced in the kidney of vitamin D$_2$-intoxicated rat were around 3–5 times higher when compared to the corresponding metabolites produced in the kidney of normal rat (Panels C and D). Further, the amount of 24(S),25,28(OH)$_3$D$_2$ produced in the kidneys of both the normal and vitamin D$_2$-intoxicated rats was about 3 times higher when compared to that of 24(R),25,26(OH)$_3$D$_2$ at all the time intervals examined. Also, the amount of 24(S),25,28(OH)$_3$D$_2$ (Panel C) was higher than that of 24(R),25(OH)$_2$D$_2$ (Panel B) itself at all time intervals studied in the kidney perfusate of vitamin D$_2$-intoxicated rats.

Metabolism of [3$\alpha$-$^3$H] 25OHD$_2$ into lipid-soluble metabolites in the kidney homogenates of normal and vitamin D$_2$-intoxicated rats

Through the kidney perfusion studies, we only analyzed the metabolites that enter into the perfusate from the kidney. It is still possible that some metabolites formed intracellularly do not enter the perfusate.
Fig. 3. Graphic representation of the \([3\alpha-^3H] 25OHD_2\) disappearance and appearance of various intermediary metabolites into the kidney perfusate. A kidney from either a normal or vitamin D\(_2\)-intoxicated rat was perfused with \([3\alpha-^3H] 25OHD_2\) (1 \(\mu\)Ci) over a time course of 5 min–4 h. Lipid extracts of perfusate samples (2 mL each) obtained at different time points were analyzed by HPLC and radioactivity was counted as described in the legend to Fig. 2. Panel A: rate of disappearance of 25OHD\(_2\) from the perfusate. Panels B, C and D: rate of appearance of the metabolites, 24(R), 25(OH)\(_2\)D\(_2\) (panel B), 24(S), 25, 28(OH)\(_3\)D\(_2\) (panel C) and 24(R), 25, 26(OH)\(_3\)D\(_2\) (panel D) into the kidney perfusate.

Therefore, we studied the metabolism of 25OHD\(_2\) in the kidney homogenates of normal and vitamin D\(_2\)-intoxicated rats. The rate of disappearance of 25OHD\(_2\) and the rate of appearance of 24(R), 25(OH)\(_2\)D\(_2\), 24(S), 25, 28(OH)\(_3\)D\(_2\) and 24(R), 25, 26(OH)\(_3\)D\(_2\) in the kidney homogenate are shown in Fig. 4. It can be seen that the amount of the substrate decreased with time in both the normal and vitamin D\(_2\)-intoxicated rats (Panel A). However, at every time interval, the amount of the unmetabolized substrate was much lower in the vitamin D\(_2\)-intoxicated rat than in the normal rat. Further, it was noted that at various time intervals, the amounts of metabolites 24(R), 25(OH)\(_2\)D\(_2\), 24(S), 25, 28(OH)\(_3\)D\(_2\) and 24(R), 25, 26(OH)\(_3\)D\(_2\) (Panels B, C and D) produced...
by the kidney homogenate of the vitamin D$_2$-intoxicated rat were much higher when compared to the corresponding metabolites produced by the kidney homogenate of normal rat. These findings are similar to our results from kidney perfusion experiments. We also noted that the amounts of 24(S),25,28(OH)$_3$D$_2$ were slightly higher when compared to 24(R),25,26(OH)$_3$D$_2$ at various time intervals studied in the kidney homogenates of both the normal and vitamin D$_2$-intoxicated rats. It is significant to note that the amounts of 24(S),25,28(OH)$_3$D$_2$ and 24(R),25,26(OH)$_3$D$_2$ produced in the kidney of vitamin D$_2$-intoxicated rat increased with the time of incubation and then subsequently decreased. This finding indicated the possibility of further metabolism of 24(R),25,26(OH)$_3$D$_2$ and 24(S),25,28(OH)$_3$D$_2$ into polar water-soluble metabolite(s).

**Metabolism of [3$\alpha$-3H] 25OHD$_2$ into polar water-soluble metabolite(s) in the kidney homogenates of normal and vitamin D$_2$-intoxicated rats**

Figure 5 shows the distribution of total radioactive counts between the water-methanol layers and methylene chloride layers from the kidney homogenates of normal and vitamin D$_2$-intoxicated rats incubated with [3$\alpha$-3H] 25OHD$_2$. It can be seen from Fig. 5 that there was no metabolism in kidney homogenates preheated at 100°C for 5 min and incubated with [3$\alpha$-3H] 25OHD$_2$ (controls). However, the total radioactive count in the methylene chloride layer was found to be significantly decreased ($p<0.001$), while it was significantly increased ($p<0.001$) in the water-methanol layer in the kidney homogenates of normal rats incubated with 250HD$_2$ when compared to their controls. Further, the total radioactive count in the methylene chloride layer was found to be significantly decreased ($p<0.001$) while it was significantly increased ($p<0.001$) in the water-methanol layer in the kidney homogenates of vitamin D$_2$-intoxicated rats incubated with 250HD$_2$ when compared to the normal rats.

**DISCUSSION**

In our present study, we first accomplished the synthesis of [3$\alpha$-3H] 250HD$_2$ enzymatically by perfusing livers isolated from vitamin D-deficient rats with [3$\alpha$-3H] vitamin D$_2$. We then used [3$\alpha$-3H] 250HD$_2$ as the substrate and demonstrated the conversion of 250HD$_2$ into 24(S),25,28(OH)$_3$D$_2$ and 24(R),25,26(OH)$_3$D$_2$ under physiological substrate concentrations in both normal and vitamin D$_2$-intoxicated rats in two in vitro systems: (a) isolated perfused kidneys and (b) rat kidney homogenates. In our previous study, 24(S),25,28(OH)$_3$D$_2$ was identified as a major renal metabolite of 250HD$_2$, indicating that C-28 hydroxylation plays an important role in the metabolism of 250HD$_2$ under physiological conditions in vitamin D$_2$-intoxicated rats (1). In the present study, 24(S),25,28(OH)$_3$D$_2$ was also identified as a major renal metabolite of 250HD$_2$ in both normal as well as vitamin D$_2$-intoxicated rats when physiological substrate concentrations were used. Thus, from our present study, it becomes obvious that C-28 hydroxylation plays a significant role in the metabolism of 250HD$_2$ even under physiological conditions. However, it is of interest to note that there was no formation of 25,28(OH)$_2$D$_2$ from [3$\alpha$-3H] 250HD$_2$. In our earlier study using pharmacological concentrations of 250HD$_2$, we also observed no formation of 25,28(OH)$_2$D$_2$, indicating that prior C-24 hydroxylation is essential for C-28 hydroxylation of 250HD$_2$ to occur (1). Thus, the structural requirement for the C-28 hydroxylation of 250HD$_2$ appears to be unique, and there is a definite sequence for the hydroxylation steps during the metabolism of this compound.

The biological activities of 24(R),25(OH)$_3$D$_2$, 24(R),25,26(OH)$_3$D$_2$ and 24(S),25,28(OH)$_3$D$_2$ are not known. However, we recently reported the biological activities of the three 1$\alpha$-hydroxylated metabolites of 24(R),25(OH)$_3$D$_2$, 24(S),25,28(OH)$_3$D$_2$ and 24(R),25,26(OH)$_3$D$_2$ ([5, 11, 12]). Both 1$\alpha$,24(R),25(OH)$_3$D$_2$ and 1$\alpha$,24(S),25,26(OH)$_3$D$_2$ were almost as active as 1$\alpha$,25(OH)$_2$D$_2$, while the C-28 hydroxylated metabolite, 1$\alpha$,24(S),25,28(OH)$_3$D$_2$ was completely inactive in inducing differentiation and inhibiting the proliferation of RWLeu-4 cells (12). Furthermore, 1$\alpha$,24(S),25,28(OH)$_3$D$_2$ was found to have no calcemic activity, suggesting that C-28 hydroxylation plays an important physiological role in the deactivation of 1$\alpha$,25(OH)$_2$D$_2$ (12). In a previous study, we demonstrated that 24(S),25,28(OH)$_2$D$_2$, the C-28 hydroxylated metabolite of 250HD$_2$, circulates in significant amounts in the vitamin D$_2$-intoxicated rat (1). Further, in the present study, the rapid formation of 24(S),25,28(OH)$_3$D$_2$ in the kidneys of the vitamin D$_2$-intoxicated rats when compared to normal rats indicates that upregulation of C-28 hydroxylation, especially during hypervitaminosis D$_2$, may play an important physiological role in the deactivation of 250HD$_2$.

In our present study, we also noted that the production of both 24(S),25,28(OH)$_3$D$_2$ and 24(R),25,
26(OH)D2 in kidney homogenates incubated with [3α-3H] 25OHD2 increased with the time of incubation and the subsequently decreased. Further the decrease in the quantity of both 24(S),25,28(OH)3D2 and 24(R),25,26(OH)3D2 in the organic phase coincided with an increase in the fraction of the total radioactivity distributed in the aqueous phase of the kidney homogenates of vitamin D2-intoxicated rats (data not shown). These findings suggested the possibility of further metabolism of 24(S),25,28(OH)3D2 and 24(R),25,26(OH)3D2 into polar water-soluble metabolite(s). We next measured the total radioactive count in the aqueous phases of kidney homogenates of both normal and vitamin D2-intoxicated rats incubated with [3α-3H] 25OHD2 for 1 h. It was noted that the amount of radioactivity in the aqueous phase of the kidney homogenates of vitamin D2-intoxicated rats is higher than that measured in the aqueous phase of normal rats, indicating the formation of higher amounts of polar water-soluble metabolite(s). We next measured the total radioactive count in the aqueous phases of kidney homogenates of both normal and vitamin D2-intoxicated rats incubated with [3α-3H] 25OHD2 for 1 h. It was noted that the amount of radioactivity in the aqueous phase of the kidney homogenates of vitamin D2-intoxicated rats is higher than that measured in the aqueous phase of normal rats, indicating the formation of higher amounts of polar water-soluble metabolite(s) for the deactivation of 25OHD2 in hypervitaminosis D2. Thus, the side chain of vitamin D2, like the side chain of vitamin D3, is metabolically susceptible to form polar water-soluble metabolite(s). Further studies are needed to find out the chemical nature of the polar metabolite(s) of the water-methanol layer. The metabolic pathway for 25OHD2 in rat kidney is depicted in Fig. 6.

In conclusion, the results of our present study provide evidence for the first time of the formation of both 24(S),25,28(OH)3D2 and 24(R),25,26(OH)3D2 under physiological conditions and the possibility of further metabolism of these two metabolites into as yet unidentified polar water-soluble metabolite(s). As the formation of all these metabolites is increased in vitamin D2-intoxicated rats when compared to normal rats, it appears that the increased rate of metabolism of 25OHD2, especially during hypervitaminosis D2, plays an important role in the inactivation of 25OHD2.

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