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BIOTRANSFORMATIONS OF 25-HYDROXYVITAMIN D_3 BY KIDNEY MICROSOMES[1]

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SUMMARY

Vitamin D_3-deficient chick kidney microsomes in vitro metabolize 25-hydroxy-[26(27)-methyl-^{3}H]-vitamin D_3 to yet structurally unidentified polar metabolites previously designated MIC-I and MIC-II. Kidney microsomes of vitamin D_3-repleted chicks could not be demonstrated to produce these metabolites when ^{3}H was the radioactive isotope in positions C-26 and C-27 of the substrate. However, when 25-hydroxy-[26,27-^{14}C]-vitamin D_3 was the radioactive substrate, MIC-I and MIC-II production was independent of the vitamin D_3 status of the chicks. These results suggest that under conditions of vitamin D_3-sufficiency, there is augmented sequential kidney metabolism of 25-hydroxyvitamin D_3 to products with modified side-chains involving C-26 and/or C-27. It is possible that this metabolism is responsible for the regulation of kidney cellular concentrations of 25-hydroxyvitamin D_3.

Vitamin D_3 undergoes sequential hydroxylation reactions first in liver microsomes (1) and then in kidney mitochondria (2,3) resulting in the formation of 25-hydroxyvitamin D_3 (25-OH-D_3)[3] and 1,25-$(OH)_2D_3$ respectively. Under certain physiologic circumstances, kidney mitochondria are also capable of hydroxylating 25-OH-D_3 on carbon 24 to yield 24,25-$(OH)_2D_3$ (4,5).

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[3]Abbreviations: 25-OH-D_3, 25-hydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; G-6-P, glucose-6-phosphate; GPDHase, glucose-6-phosphate dehydrogenase (Sigma Type VII).

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The physiological circumstances associated with the selective formation of these hydroxylated metabolites have been investigated extensively (6). It is known, for example, that during vitamin D_3 depletion or during deviations of serum calcium and phosphate concentrations from normalcy (7,8), the synthesis of 24,25-$(OH)_2D_3$ is suppressed and the synthesis of 1,25-$(OH)_2D_3$ is enhanced from the common precursor substrate 25-OH-D_3. A complete understanding of the molecular events associated with the regulation of the selective synthesis of these metabolites remains elusive. More recently, another pathway for the metabolism of 25-OH-D_3 in the kidney has been established (9). Kidney microsomes from vitamin D_3-deficient chicks have been shown to convert 25-OH-D_3 to detectable but yet unidentified products, tentatively designated MIC-I and MIC-II (9). Their formation appears to involve monooxygenase catalyzed reactions mediated by cytochrome P-450 (10). The purpose of this communication is to present evidence that the biotransformation of 25-OH-D_3 by kidney microsomes is dependent on the vitamin D status of the animal and may involve side-chain modification.

MATERIALS AND METHODS

Animals: One-day old white Leghorn cockerel chicks were obtained from Northern Hatcheries, Beaver Dam, WI., and maintained on a vitamin D-deficient rachitogenic test diet (U.S. Biochemicals, Cleveland, OH) containing 1% phosphorus and supplemented with $CaCO_3$ to contain 3% calcium.

Incubations: For the biosynthesis of 24,25-$(OH)_2$-[26(27)-methyl-^{3}H]-vitamin D_3, a 20% chick kidney homogenate was prepared in the following solution: 15 mM Tris-acetate, 0.19 M sucrose, 3.7 mM malate, 1.87 mM magnesium acetate, 0.15 mM NADP and 1.7 mM G-6-P. To 6 ml aliquots of the homogenate in 125 ml Erlenmeyer flasks containing 8.4 units of GPDHase, the substrate 25-OH[26(27)-methyl-^{3}H]-vitamin D_3, 10 Ci/mmol, was added in 50 µl of 95% ethanol. The flasks were gassed with 100% oxygen for one minute, then incubated for 90 minutes at 37°. The preparation of microsomes and the microsomal incubations were as described previously (10). Extractions and Sephadex LH-20 column chromatography of all incubations were performed by previously published procedures (10,11). Protein determinations were by the method of Lowry, et. al (12).

High Pressure Liquid Chromatography (HPLC): HPLC was performed on a Glenco Modular Component HPLC system fitted with model SVOV-6-1 injection valve. Using such a system, injection of 100 µl samples is made without interruption of solvent flow at an operating pressure of 500 psi. A single Du Pont Zorbax-Sil column (4.6 mm x 22 cm) was used. The solvent system was 10% isopropanol in n-hexane. Fifty fractions 0.5 ml in volume were collected at a flow rate of 2.5 ml/minute (13).

RESULTS

It has been shown that administration of 1,25-$(OH)_2D_3$ to vitamin D_3-deficient chicks will activate the renal mitochondrial 24-hydroxylase (14). In the course of utilizing this approach to biosynthesize 24,25-$(OH)_2D_3$ using kidney homogenates, it was found that the 24,25-$(OH)_2D_3$ product isolated from Sephadex LH-20 columns was heterogeneous when purified by HPLC. The results are shown in Fig. 1, panels A and B. Similar results were obtained from birds dosed identically with 24,25-$(OH)_2D_3$. However, when the birds were dosed with the same amounts of 25-OH-D_3, the heterogeneity seen in panel B was completely abolished. Only a single homogeneous peak of 24,25-$(OH)_2D_3$ was seen in the HPLC profile (Fig. 1, panels C and D).

To explore the possibility that the source of this heterogeneity is microsomal in origin, identical incubations using kidney homogenates from 1,25-$(OH)_2D_3$-dosed chicks were performed in the absence of G-6-P, NADP and GPDHase (see Methods). In this system in which malate is the sole source of the reducing equivalents, the products of the incubations should be exclusively mitochondrial in origin. The results of such incubations are shown in Fig. 1, panels E and F. A single homogeneous peak of 24,25-$(OH)_2D_3$ was produced as expected. Evidence for the purity of the 24,25-$(OH)_2D_3$ produced in the above experiments was obtained by the periodate oxidation procedure (15). The results are shown in Table I. Whenever homogeneous products were suspected (Fig. 1, panels C, D, E and F), essentially complete loss of tritium (>92%) was observed as expected from a pure metabolite with vicinal hydroxyl groups. The peaks in Fig. 1, panels A and B, showed decreased tritium losses after periodate oxidation. These data show that none of the products in panels A and B is composed entirely of 24,25-$(OH)_2D_3$. It should be noted that periodate sensitivities of isolated MIC-I and MIC-II were 34.7 and 26.1% respectively, values considerably lower than those for products "b" and "c" observed in panel B of Fig. 1. This discrepancy could possibly be explained by contamination of these peaks by the product in peak "a" suspected to be 24,25-$(OH)_2D_3$.

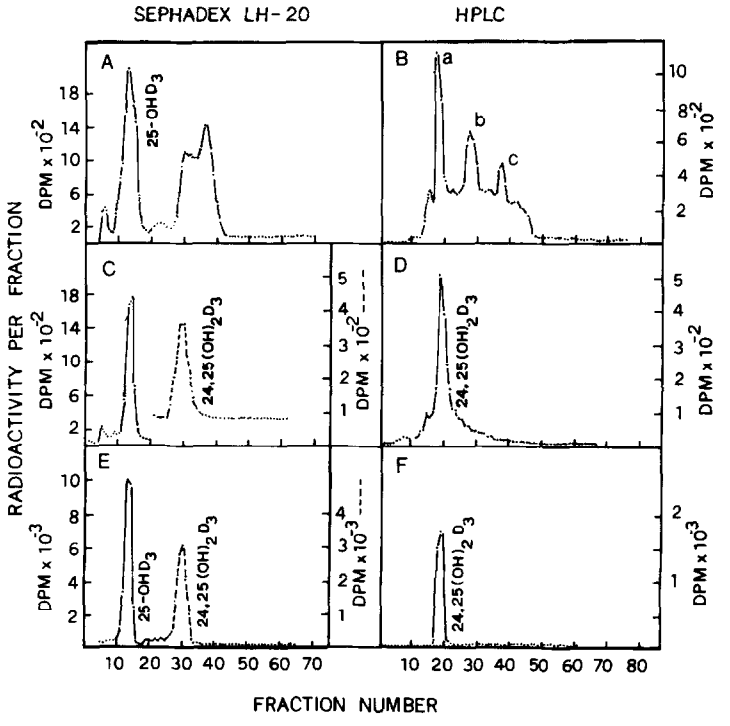

Figure 1. One-day old chicks were dosed orally for 6 days with 326 pmol of either vitamin D_3, 25-OH-D_3, 24,25-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$ in 100 µl of Wesson oil. They were maintained on the rachitogenic diet with free access to distilled water until killing by cervical dislocation. Their kidneys were immediately removed and chilled in the solution described under Methods. Kidney homogenates or microsomes were also prepared as described under Methods. Chromatography on Sephadex LH-20 was performed using 20 g of Sephadex in 65:35 chloroform:n-hexane. Five ml fractions were collected. An aliquot was taken from each fraction, evaporated to dryness under forced air and radioactivity determined as previously reported (10). The fractions containing the products were pooled, evaporated to dryness and dissolved in 10% isopropanol in n-hexane. A 100 µl aliquot was used for HPLC.

It has been suggested that the products of the microsomal metabolism of 25-OH-D_3 may involve side-chain modification (9). To further explore this possibility, 25-OH-D_3 substrate radiolabeled with carbon-14 or with tritium was incubated in experiments utilizing isolated microsomes from kidneys of chicks treated as described in the legend in Fig. 1. In these incubations with isolated microsomes, no 24,25-$(OH)_2D_3$ is produced in the absence of mitochondria. Hence, the microsomal products of 25-OH-D_3 can be readily

TABLE I

PERIODATE OXIDATION OF PRODUCTS OBTAINED FROM SEPHADEX LH-20 AND HPLC CHROMATOGRAMS SHOWN IN FIG. I

	% ^{3}H LOSS FROM PRODUCT*	
TREATMENT	Sephadex LH-20	HPLC
1,25-$(OH)_2D_3$ Dosed (Complete incubation medium)	87	a) 79 b) 72 c) 39
25-OH-D_3 Dosed (Complete incubation medium)	92	99
1,25-$(OH)_2D_3$ Dosed (Malate only)	96	95

* Periodate oxidation was performed according to published procedures (15). The tritium in the substrate is only in positions 26(27), hence, the vicinal 24 hydroxylation of 25-OH-D_3 would yield a product with quantitative susceptibility to periodate cleavage and loss of radioactivity.

separated on glass columns containing 20 g Sephadex LH-20 (9). The microsomal products have elution profiles similar to the products shown in Fig. 1, panel A but with two distinctly separated peaks of radioactivity (9). The results of the above experiments are tabulated in Table II. These results show that when ^{14}C-25-OH-D_3 is the substrate, the formation of the microsomal products, designated MIC-I and MIC-II, is independent of the vitamin D_3 status of the chicks. In contrast, when ^{3}H-25-OH-D_3 is the substrate, significant amounts of microsomal products are seen only in completely D-deficient birds. However, in the birds given vitamin D_3 or 25-OH-D_3, no microsomal products can be detected when ^{3}H-25-OH-D_3 is the substrate while in comparable assays the MIC-I and MIC-II activities are unchanged when ^{14}C-25-OH-D_3 is the substrate. In 6-day old marginally depleted birds, MIC - I activity appears to persist with the tritiated substrate. It is interesting to note, however, that contrary to the effect of 25-OH-D_3 or

TABLE II

MICROSOMAL INCUBATION PRODUCTS QUANTITATED BY SEPHADEX LH-20 CHROMATOGRAPHY

TREATMENT*	PRODUCT+ (pmol/hr/mg protein)			
	MIC-I		MIC-II	
	^{3}H Substrate	^{14}C Substrate	^{3}H Substrate	^{14}C Substrate
5-week Old Deficient	14.2	23.5	23.5	35.2
6-day Old Oil Dosed	4.4	14.9	0	21.1
Vitamin D_3 Dosed	0	14.1	0	25.9
25-OH-D_3 Dosed	0	12.6	0	25.8
24,25-$(OH)_2D_3$ Dosed	3.4	-	0	-
1,25-$(OH)_2D_3$ Dosed	4.3	-	0	-

* 325 pmol of metabolite in 100 µl Wesson oil orally for 6 days as described in Fig. 1 legend.

+ Isolated kidney microsomes were incubated and the products analyzed as described previously (10) using either 25-OH-[26(27)-methyl-^{3}H]-vitamin D_3 (10 Ci/mmol) or 25-OH-[26,27-^{14}C]-vitamin D_3 (40 mCi/mmol) as the substrate. The data represent the average of duplicate assays.

vitamin D_3, neither 1,25-$(OH)_2D_3$ nor 24,25-$(OH)_2D_3$ abolishes the persistence of MIC-I. This appears to be, at least in part, a specific function of 25-OH-D_3. The lowered activities measured with the ^{3}H-25-OH-D_3, undoubtedly, represent partial loss of tritium from C-26 and/or C-27 of the substrate.

DISCUSSION

The metabolism of 25-OH-D_3 in the kidney is subject to pathways other than those committed to the production of 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ by the mitochondria. Kidney microsomes metabolize 25-OH-D_3 to more polar compounds (9). Specific information concerning these alternate pathways was obtained by comparison of the microsomal products formed from 25-OH-D_3 radiolabeled with either ^{3}H or ^{14}C in positions 26 and 27 of the molecule. Under conditions of complete vitamin D_3 deficiency, there is accumulation of microsomal products of identical mobilities arising from either the ^{3}H or ^{14}C labeled substrate. However, in conditions approaching vitamin D_3 sufficiency there appears to be significantly less detectable products with the ^{3}H substrate than with the ^{14}C substrate. This would be expected if during vitamin

D repletion there is augmented conversion of the accumulated products seen in deficiency to further as yet undetected product(s) herein designated "X". Thus, under conditions of vitamin D_3 deficiency, conversion of MIC-II to "X" is abolished allowing accumulation of MIC -I as well as the relatively ^3H-poor MIC-II to detectable levels. In contrast, conversion of MIC-II to "X" is augmented during vitamin D_3 repletion which may prevent MIC-II accumulation to detectable levels. The apparent absence of the product MIC-II from the ^3H-25-OH-D_3 precursor even in the studies of the marginally depleted birds would suggest that the ^{14}C substrate is a much more sensitive indicator of product formation. Possibly, incubations containing microsomes from vitamin D_3 repleted birds and large amounts of 26,27-^3H substrate are likely to produce MIC-II that can be detected upon chromatography. In this regard, the recent availability of 25-hydroxy-[23,24-^3H]-vitamin D_3 may prove to be useful in evaluating the extent of the side-chain modification.

The relative contribution of the microsomal pathways to the overall metabolic fate of 25-OH-D_3 is currently being investigated. It is interesting to note that the cytochrome P-450 or b_5 level of kidney microsomes is independent of the vitamin D_3 status of the chicks (10). This observation argues against variations in enzyme levels being responsible for the above reported results. Hence, it is possible that microsomal pathways are responsible for the regulation of kidney cell concentrations of 25-OH-D_3.

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