KIDNEY MICROSOMAL METABOLISM OF 25-HYDROXYVITAMIN D_3[+]

by

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Vitamin D_3-deficient chick kidney microsomes *in vitro* metabolize 25-hydroxyvitamin D_3 to two polar metabolites by a pathway which may involve side-chain modification. Molecular oxygen and a source of reduced nicotinamide adenine dinucleotide phosphate are required for this metabolism. Kidney cytosol obtained from deficient chicks or kidney microsomes of vitamin D_3-repleted chicks do not metabolize 25-hydroxyvitamin D_3. The two products are tentatively designated MIC-I and MIC-II.

The hydroxylations of vitamin D_3 to the more polar metabolites 25-hydroxyvitamin D_3 (25-OH-D_3)[1] and 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) are prerequisites for function of the vitamin at physiologic concentrations (1). The initial activation of vitamin D_3 to 25-OH-D_3 occurs in liver and has been shown to require molecular oxygen and reduced pyridine nucleotides (2). The subsequent conversion of 25-OH-D_3 to 1,25-$(OH)_2D_3$ occurs exclusively in kidney mitochondria catalyzed by a cytochrome P-450 dependent multi-component system (3, 4). This system also requires molecular oxygen and reduced pyridine nucleotide (5).

The *in vivo* oxidative cleavage of the 25-OH-D_3 and 1,25-$(OH)_2D_3$ side-chains to yet unknown products plus carbon dioxide is also known (6-8). Nephrectomy and conditions suppressing 25-OH-D_3-1α-hydroxylase activity in

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[1]Abbreviations: 25-OH-D_3, 25-hydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DTT, 1,4-dithiothreitol; EDTA, ethylene-diaminetetraacetate, disodium salt.

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the kidney abolished the production of $^{14}CO_2$ from 25-OH-[26,27-^{14}C]D_3 but not from 1,25-$(OH)_2D_3$. Thus a pathway of vitamin D metabolism involving oxidative cleavage of the side-chain of 1,25-$(OH)_2D_3$ which occurs *in vivo* is clearly known.

The present report describes a kidney microsomal enzyme system capable of metabolizing 25-OH-D_3. A rachitic state (elevated 25-OH-D_3-1α-hydroxylase activity) is found to be necessary for the *in vitro* expression of this metabolism.

MATERIALS AND METHODS

One-day old white Leghorn cockerel chicks (Northern Hatcheries, Beaver Dam, Wis.) were maintained on a vitamin D-deficient purified soy protein diet (9) for 4 weeks before use. The chicks were killed by decapitation. The kidneys were removed and rinsed with chilled buffer (0.25 M sucrose - 15 mM Tris-acetate, pH 7.4, 1 mM DTT and 10^{-4} M EDTA), then homogenized in 4 volumes (w/v) of the buffer using an ice-cold Potter-Elvehjem homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 500 g (700 rpm in a Sorvall SS-34 rotor) at 4° for 10 min. The postmitochondrial supernatant was then subjected to centrifugation at 20,000 rpm in a Beckman Model L preparative ultracentrifuge at 4° for 30 min using a 30 rotor. The pellet was discarded. The centrifugation was repeated and the pellet discarded again. The supernatant was then centrifuged at 30,000 rpm in the Beckman Model L ultracentrifuge at 4° for 90 min. The supernatant was generally discarded. The red microsomal pellet was suspended in the buffer with gentle homogenization to a protein concentration of 16 mg/ml as determined by the Lowry method (10).

Incubations were carried out using 1.5 ml final volume in 25-ml Erlenmeyer flasks containing 1 ml of the microsomal preparation (16 mg protein), 5 mM magnesium acetate, 5 mM glucose-6-phosphate, 0.5 mM NADP and 500 ng radiolabeled substrate[2] in 5 µl of absolute ethanol. The substrate used was either 25-OH-[26,27-^{3}H]D_3 with a specific radioactivity of 100 dpm/ng, or 25-OH-[26,27-^{14}C]D_3 also with a specific radioactivity of 100 dpm/ng. The flasks were gassed with 100% O_2 for 1 min then corked. The reactions were initiated by the addition of 1.5 units of yeast glucose-6-phosphate dehydrogenase (Sigma Type VII) in 10 µl of 3.2 M ammonium sulfate. After 15 min at 37° with gentle agitation, the reactions were terminated by the addition of 10 ml of methanol-chloroform (2:1 v/v). Extraction and chromatography of the reaction products on columns containing 15 g Sephadex LH-20 were as previously described (5). Radioactivity was counted at an efficiency of 50% for ^{3}H and 80% for ^{14}C.

RESULTS

The Sephadex LH-20 chromatography of the incubation extracts is shown in Figure 1. Kidney microsomes prepared and incubated as described converted about 6% of the 25-OH-D_3 to MIC-I and an additional 10% to MIC-II as detectable

[2]Calculated from the UV spectrum of stock solutions in 95% ethanol using a value for ε_{264} of 18,200 M^{-1} cm^{-1}.

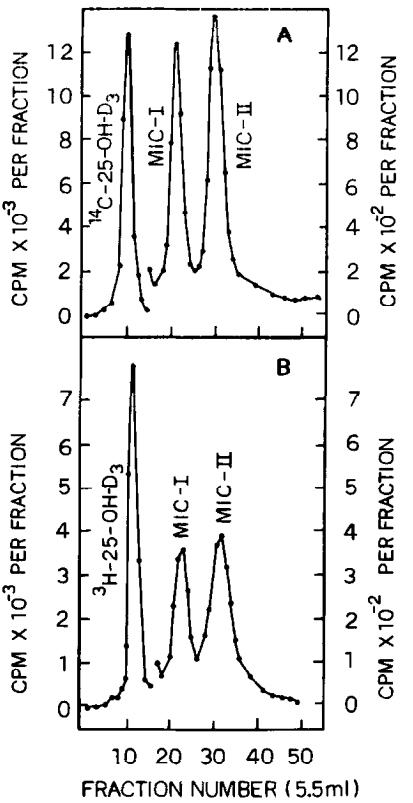

Figure 1. Sephadex LH-20 chromatographic profiles of extracts from microsomes incubated with either 25-OH-[26,27-^{14}C]D$_3$ or 25-OH-[26,27-^{3}H]D$_3$. The columns containing 15 g of Sephadex were equilibrated and eluted with chloroform:n-hexane (65:35 v/v). Elution rate was 1 ml/min. Panel A represents chromatography of the extracts when 25-OH-[26,27-^{14}C]D$_3$ was the substrate and panel B when 25-OH-[26,27-^{3}H]D$_3$ was the substrate. The scale on the right ordinate applies to products MIC-I and MIC-II only.

products when 25-OH-[26,27-^{3}H]D$_3$ was the tracer used. However, the conversions were about 10% and 15% respectively when 25-OH-[26,27-^{14}C]D$_3$ was the tracer. The consistent variation in these radioactivity proportions in the chloroform extractable components could suggest a partial loss of tritium from the substrate side-chain. However, other modifications in the remaining portions of the substrate molecule cannot be excluded. Consistent with the interpretation of side-chain modification is the observation that 80% of the total tritium is generally recovered in the lipid phase compared to the 90% found

Table I. Requirements for the Kidney Microsomal 25-OH-D_3 Metabolism

Assay contents	pmole 25-OH-[26,27-^{3}H]D_3/hr/mg protein^{-1} appearing as MIC-I	MIC-II
Complete with oxygen[a]	19.4	32.5
Boiled microsomes[b]	0	0
- Microsomes	0	0
- Magnesium	17.5	30.6
- NADP + GPDH[c]	0	0
+ NADP - GPDH	0	0
+ NADH[d]	Trace	Trace
+ NAD - ADH	0	0
Complete with air	19.0	31.6
Complete with 100% nitrogen	Trace	Trace

[a]The complete assay medium is given under "Methods." Reaction volume 1.5 ml.

[b]Treated at 100° (boiling water bath) for 10 min.

[c]Glucose-6-phosphate dehydrogenase.

[d]A generating system consisting of 0.5 mM NAD, 1.5 units of yeast alcohol dehydrogenase (ADH) and the 5 µl of absolute alcohol present from the addition of the radiolabeled substrate.

in the case of ^{14}C. Although one cannot presently exclude the involvement of isotope effect in these findings, it is important to note that the analogous vitamin D-deficient kidney mitochondrial 25-OH-D_3-1α-hydroxylase activity is not affected by the type of isotope present in the substrate 25-OH-D_3.

The role of oxidized and reduced pyridine nucleotides in the kidney microsomal metabolism of 25-OH-D_3 is shown in Table I. It is clear that NADPH is specifically required for the apparent protein-dependent microsomal activity. In addition, as shown in Table I there is a total dependence on the presence

of molecular oxygen. Under complete anaerobic conditions produced as previously described (5), neither MIC-I nor MIC-II is formed in measurable quantity.

DISCUSSION

Early studies with isolated rachitic chick kidney microsomes failed to reveal detectable metabolism of 25-OH-D_3 by this cellular component (11). In addition, the presently described metabolites have not been detected in chick kidney homogenate incubations. Several factors such as inappropriate substrate for generation of NADPH, insufficient amounts of microsomes and low specific activity of substrate making detection difficult may have contributed to this failure. The present report shows clearly that kidney microsomes are capable of metabolizing 25-OH-D_3 even though the activity is rather small compared to the 25-OH-D_3-1α-hydroxylase of kidney.

The molecular oxygen and reduced pyridine nucleotide requirements of the kidney microsomal system suggest that the substrate is oxidatively metabolized. The kinetics of appearance of the two metabolites MIC-I and MIC-II have not been determined. Furthermore, whether carbon dioxide is oxidatively produced from subsequent interconversions of these metabolites is not clear. Attempts to trap $^{14}CO_2$ on filter papers saturated with 20% KOH placed in the center wells of sealed Warburg flasks used as reaction vessels instead of the usual Erlenmeyer flasks failed to demonstrate $^{14}CO_2$ evolution. However, in view of the amount of products formed, and of the unknown extent to which each product might give rise to CO_2 formation, the oxidative cleavage of the 25-OH-D_3 side-chain in this system still remains a possibility.

The significance of this metabolic pathway in the overall vitamin D_3 metabolism is unknown; but in view of its specific requirements it is possible that it may be of significance *in vivo*.

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