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Rat Kidney Microsomes Convert 25-Hydroxyvitamin D_3 to an Unidentified Metabolite

Myrtle THIERRY-PALMER, T. Kenney GRAY, Mary E. WILLIAMS, and Roy V. TALMAGE

Departments of Medicine, Pharmacology, and Surgery, School of Medicine, University of North Carolina Chapel Hill, North Carolina 27514

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Summary: Kidney microsomes from vitamin D-deficient rats and from thyroparathyroidectomized rats converted 25-hydroxyvitamin D_3 to an unidentified metabolite. The addition of the cytosolic fraction enhanced microsomal synthesis of this metabolite two-fold. The kinetics of the conversion in the presence of the cytosolic fraction was allosteric, suggesting that the enzyme responsible for synthesis of this metabolite might serve some role in the regulation of vitamin D metabolism. Microsomes from vitamin D-fed thyroparathyroidectomized rats also produced a second metabolite, tentatively identified as 25,26-dihydroxyvitamin D_3 because of its comigration with 25,26-dihydroxyvitamin D_3 in three different chromatographic systems.

Although the kidney is known to be an important site in the metabolism of 25-hydroxyvitamin D_3 (1) only one paper (2) has appeared in which metabolism of $25OHD_3$ *in vitro* by rat kidney microsomes has been studied. It was reported that kidney microsomes isolated from rats fed a vitamin D sufficient diet converted $25OHD_3$ to 25,26-dihydroxyvitamin D_3. Chick kidney microsomes (3-5) convert $25OHD_3$ *in vitro* to two unidentified polar metabolites (Mic-I and Mic-II). Cytochrome P-450 was shown to be an integral component of this metabolism. We report here on the conversion of $25OHD_3$ by rat kidney microsomes *in vitro* to an unidentified metabolite. Microsomes from vitamin D-fed thyroparathyroidectomized rats also produced a second metabolite, tentatively identified as $25,26(OH)_2D_3$ because of its comigration with synthetic $25,26(OH)_2D_3$ in three different chromatographic systems.

MATERIALS AND METHODS

Male Sprague Dawley rats (ARS, Madison WI) were maintained on a diet modified from Boass *et al* (6) containing 5 IU vitamin D_3 per gram with calcium and phosphorus present at a level of 0.4% and 0.35%. Vitamin D-deficient rats were

Abbreviations used: $25OHD_3$, 25-hydroxyvitamin D_3; $24,25(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; $25,26(OH)_2D_3$, 25,26-dihydroxyvitamin D_3.

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nursed from a mother fed an identical diet which lacked vitamin D and were weaned to a diet without vitamin D. They were sacrificed at 150g body weight and had calcium levels of 5.5-6.0 mg percent. The thyroid-parathyroid complex was removed from vitamin D-fed rats (250g body weight) twenty four hours before sacrifice. During this period the thyroparathyroidectomized rats were provided with drinking water containing 2% calcium lactate and 10% glucose.

Rats were anesthetized with ether, bled by cardiac puncture, and their kidneys excised. A 10% homogenate of their kidneys was prepared in 0.25 M sucrose, 0.05 M Tris-acetate (pH 7.4) using a motor driven glass-teflon Potter-Elvehjem homogenizer. Mitochondria and microsomes were isolated (7), washed, and resuspended in a volume of 0.25 M sucrose equal to the original volume of the homogenate.

Incubation flasks (25 ml erlenmeyers) contained one ml of microsomal suspension (and in some cases one ml of the cytosolic fraction), 2.0 mM magnesium acetate, 25 mM potassium acetate, 0.4 mM $NADP^+$, 20 mM nicotinamide, 4.0 mM glucose 6-phosphate, 1.2 units glucose 6-phosphate dehydrogenase, 170 mM sucrose and 17 mM Tris-acetate (pH 7.4) in a total volume of 3.0 ml. The flasks were flushed with oxygen for one min and corked. Two µg of $25OHD_3$ (100,000 cpm of 25-hydroxy[26,27-^{3}H]vitamin D_3, 19 Ci/mmol, Amersham, Arlington Heights, IL) were added in 20 µl ethanol at zero time and the flasks were incubated in a Dubnoff metabolic shaker for 20 min at 30°C. The reaction was halted by immediate immersion of the flasks into an ethanol-dry ice bath. Mitochondria (one ml suspension) were incubated with 25 mM succinate, 2 mM magnesium acetate, 17 mM Tris-acetate, and 170 mM sucrose for 30 minutes at 37°C. Substrate levels were the same as that used for microsomes. The incubation mixtures were extracted once with 3 volumes of ether (passed first over an alumina column) and twice with an equal volume of cyclohexane/ethylacetate (8). The extracts were evaporated under nitrogen and chromatographed by high pressure liquid chromatography (Laboratory Data Control, Riviera Beach, FL) on a Zorbax-Sil column (Dupont, Wilmington, DE) using 6% isopropanol in hexane at a flow rate of 1 ml/min (9) or 2% methanol in dichloromethane at a flow rate of 0.5 ml/min (10). Radioactivity was monitored by collecting and counting one minute fractions from the column. The liquid scintillation fluid was 4a20 (Research Products International, Elk Grove Village, IL) and the counter was Beckman Model LS-230. In some experiments samples were initially purified by thin layer chromatography on silica gel 60 (MC/B Manufacturing Chemists, Cincinnati, OH) with chloroform/ethylacetate (1/1,v/v). The region on the TLC plate corresponding to the two metabolites was extracted into ethylacetate, evaporated under nitrogen, and rechromatographed on the HPLC systems above. Protein was determined by the method of Lowry (11).

RESULTS

The metabolism of [^{3}H]$25OHD_3$ by microsomes and cytosol from the kidneys of thyroparathyroidectomized rats is illustrated in Figure 1. Two polar metabolites were produced. The major component, x, migrated between $24,25(OH)_2D_3$ and $25,26(OH)_2D_3$ on a straight phase HPLC column developed with hexane/isopropanol (94/6,v/v). The minor metabolite, y, comigrated with synthetic $25,26\text{-}(OH)_2D_3$ on this system. The relative positions of metabolite x changed (Fig. 2A) when it was rechromatographed with dichloromethane/methanol (98/2, v/v), but the relative position (Fig. 2B) of metabolite y was unchanged. Chromatography by HPLC was sometimes preceded by thin layer chromatography on

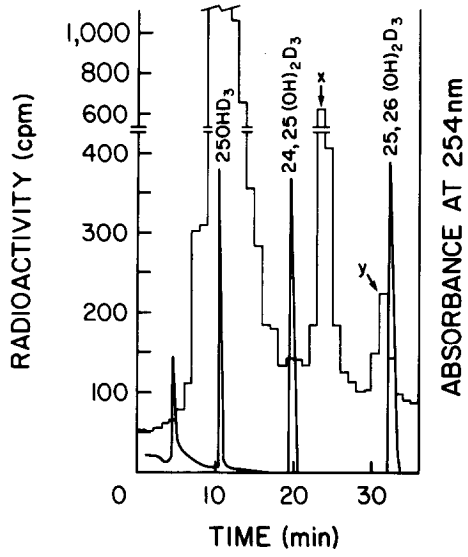

Figure 1. Microsomes and the cytosolic fraction from vitamin D-fed thyroparathyroidectomized rats were incubated as described in Materials & Methods. The incubation mixtures were extracted for lipids and the lipids were chromatographed on a normal phase HPLC column with hexane/isopropanol (94/6, v/v). The histogram shows the elution pattern of the radioactive metabolites; the line graph shows the elution pattern of synthetic standards as determined by absorbance at 254 nm.

silica gel with chloroform/ethylacetate. In this system, metabolite x comigrated with synthetic $24,25(OH)_2D_3$ and metabolite y with synthetic $25,26(OH)_2D_3$.

The microsomes contained the synthetic activity for production of the metabolites, but addition of the cytosolic fraction to the incubation mixture

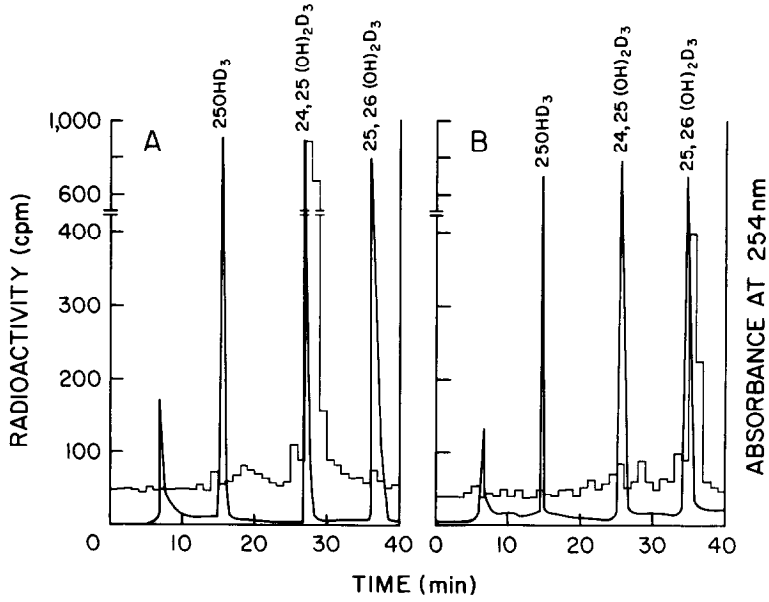

Figure 2. Metabolites x (Fig. 2A) and y (Fig. 2B) were rechromatographed on a normal phase HPLC column with dichloromethane/methanol (98/2, v/v).

TABLE 1. Subcellular Location of Enzyme Activity for Conversion of $25OHD_3$ to Metabolites x and y.

Subcellular Fraction	x,pmoles mg protein/min Mean ± S.D.	y,pmoles/ mg protein/min Mean ± S.D.
Microsomes	3.20 ± 0.06	2.83 ± 0.51
Cytosolic fraction	0	0
Microsomes + cytosolic fraction	7.22 ± 0.27	2.34 ± 0.15
Mitochondria	0	0

Thyroparathyroidectomized rats were killed 24 hours after surgery. Microsomes, mitochondria, and cytosol were incubated as described in Materials & Methods. The lipid extracts of the incubation mixtures were chromatographed on a normal phase column with hexane/ isopropanol (94/6,v/v). Values are mean ± S.D. for 3-4 incubations.

enhanced synthesis of metabolite x by a factor of two (Table 1). Synthesis of putative $25,26(OH)_2D_3$ was unaffected by the cytosolic fraction. Microsomes, in the presence of the cytosolic fraction, converted more of the $25OHD_3$ substrate to metabolite x than to metabolite y; 2.95 ± 0.11 percent of the radioactivity recovered from the microsomes and cytosol migrated with metabolite x and 0.95 ± 0.06 percent migrated with metabolite y. Metabolites x and y were not produced by renal mitochondria incubated with magnesium acetate, succinic acid, and levels of $25OHD_3$ identical to those used for incubation of renal microsomes.

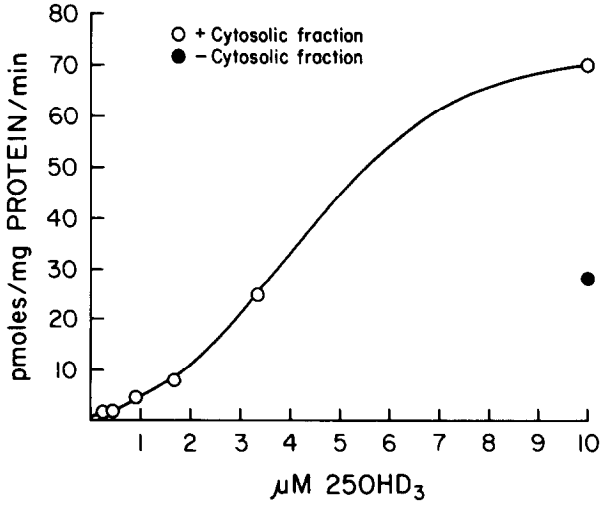

Figure 3. Effect of substrate concentration on the rate of formation of metabolite x by microsomes in the presence of cytosol. Microsomes from the kidneys of vitamin D-deficient rats were incubated as described in Materials & Methods, but with varying quantities of substrate.

The kinetics of formation of metabolite x by microsomes and cytosol from vitamin D-deficient rats was allosteric (Fig. 3). Metabolite x was first observed when microsomes and cytosol from thyroidectomized rats were incubated with [^{3}H]25OHD$_3$. It has been consistently synthesized by microsomes and cytosol from thyroidectomized rats (28 of 28 thyroparathyroidectomized or thyroidectomized rats with parathyroid transplants), but only occasionally synthesized by microsomes and cytosol from thyroid-intact rats (5 of 25). More frequently, [^{3}H]25OHD$_3$ was not further metabolized by microsomes and cytosol from thyroid-intact rats.

DISCUSSION

Data published here demonstrate that rat kidney microsomes convert 25OHD$_3$ to two metabolites. Metabolite x does not migrate with any known vitamin D metabolite when chromatographed on a normal phase high pressure liquid chromatography column using hexane/isopropanol. Metabolite x is consistently produced by microsomes of thyroidectomized rats, but not by intact vitamin D deficient rats or vitamin D replete rats. The kinetics of formation of metabolite x by vitamin D deficient rats is allosteric. For such a regulatory enzyme, a negative modulator could interact with the enzyme, increasing the substrate concentration required to produce metabolite x. This modulator might be absent in the thyroidectomized rats, but definitive experiments have not yet been done to elucidate this phenomenon. Alternatively, some positive modulator (not always present in the thyroid-intact rat) might decrease the substrate concentration required to produce metabolite x. A role for metabolite x has not yet been determined, but its synthesis by a regulatory enzyme would suggest that it is an important intracellular metabolite. The enzyme responsible for its synthesis may be at a control point in the intermediary metabolism of vitamin D$_3$.

Metabolite y migrates in three different chromatographic systems with synthetic 25,26(OH)$_2$D$_3$. Napoli et al (2) have identified 25,26(OH)$_2$D$_3$ as a rat renal 25OHD$_3$ metabolite so that this result would be consistent with their findings. It must be pointed out here that if metabolite y is 25,26(OH)$_2$D$_3$, it is produced in the microsomes at a higher level than that apparent from

quantitation of the radioactive peak. Our $25OHD_3$ was labeled with tritium in positions 26 and 27 so that the specific activity of the $25,26(OH)_2D_3$ product would have been less than half that of the starting $25OHD_3$ (12).

Work is in progress in our laboratory on the identity and function of metabolite x. A knowledge of the identity and function of metabolite x should provide a better understanding of the regulatory mechanisms involved in vitamin D_3 metabolism in the rat.

REFERENCES

1. Gray, R.W., Boyle, I.T., and DeLuca, H.F. (1971) Science 172,1232-1234.
2. Napoli, J.L., Okita, R.T., Master, B.S., and Horst, R.L. (1981) Biochemistry 20,5865-5871.
3. Ghazarian, J.G., and DeLuca, H.F. (1977) Biochem. Biophys. Res. Commun. 75,550-555.
4. Ghazarian, J.G., Hsu, P.Y., and Peterson, B.L. (1977) Arch. Biochem. Biophys. 184,596-604.
5. Gray, R.W., Caldas, A.E., Weber, J.L., and Ghazarian, J.G. (1978) Biochem. Biophys. Res. Commun. 82,121-128.
6. Boass, A., Toverud, S.U., McCain, T.A., Pike, J.W., and Haussler, M.R. (1977) Nature 267,630-632.
7. Wecksler, W.R., Henry, H.L., and Norman, A.W. (1977) Arch. Biochem. Biophys. 183,168-175.
8. Bouillon, R., DeMoor, P., Baggiolini, E.G., and Uskokovic, M.R. (1980) Clin. Chem. 26,562-567.
9. Jones, G., and DeLuca, H.F. (1975) J. Lipid Res. 16,448-453.
10. Ikekawa, N., and Koizumi, N. (1976) J. Chromatogr. 119,227-232.
11. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193,265-275.
12. Tanaka, Y., and DeLuca, H.F. (1982) Arch. Biochem. Biophys. 213,517-522.