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SYNTHESIS AND FUNCTION OF 8α,25-DIHYDROXY-3-OXONEOCHOLECALCIFEROL IN LIVER

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Summary—25-Hydroxycholecalciferol (25-OHD_3) is converted to 8α,25-dihydroxy-3-oxoneocholecalciferol [8,25-$(OH)_2$-3-oxoneo-D_3] by liver microsomes, alveolar macrophages and myeloid leukemia cells. The characteristics of this reaction in liver microsomes have been determined. Omission of an NADPH-generating system or NADH resulted in a >75% reduction in the production of 8,25-$(OH)_2$-3-oxoneo-D_3. In the absence of the cytosolic fraction, 25-OHD_3 was converted to products that comigrated with 8,25-$(OH)_2$-3-oxoneo-D_3 on a silica column developed with hexane–isopropanol, thereby preventing quantitation. Production of 8,25-$(OH)_2$-3-oxoneo-D_3 was unaffected by EDTA and was stimulated by *N,N'*-diphenyl-*p*-phenylenediamine. Both progesterone and pregnenolone inhibited production of 8,25-$(OH)_2$-3-oxoneo-D_3; inhibition by progesterone was greater than that by pregnenolone. 8,25-$(OH)_2$-3-Oxoneo-D_3 did not bind the thymus receptor for 1,25-dihydroxycholecalciferol [1,25-$(OH)_2D_3$] at concentrations 10-fold higher than that of 1,25-$(OH)_2D_3$. The lack of affinity of 8,25-$(OH)_2$-3-oxoneo-D_3 for the 1,25-$(OH)_2D_3$ receptor suggests that this metabolite is a degradative product of 25-OHD_3, which might be produced when 25-OHD_3 concentrations in the liver are excessive. Synthesis of this metabolite in the liver may be catalyzed by enzymes that also metabolize other steroids.

INTRODUCTION

25-Hydroxycholecalciferol (25-OHD_3) is converted to 8α,25-dihydroxy-3-oxoneocholecalciferol (8α,25-dihydroxy-9,10-seco-4,6,10(19)-cholestatrien-3-one; 8,25-$(OH)_2$-3-oxoneo-D_3) by alveolar macrophages [1, 2], myeloid leukemia (M1, HL-60, and U937) cells [1, 2], and liver microsomes [3]. 8,25-$(OH)_2$-3-Oxoneo-D_3 results from hydroxylation at the 8 position and oxidation at position 3. The subsequent shift in double bonds changes the absorption maximum from 265 nm for 25-OHD_3 to 295 nm [1, 3] for the 8-hydroxy-3-keto metabolite.

8,25-$(OH)_2$-3-Oxoneo-D_3 was first identified as a product of 25-OHD_3 metabolism by phagocytic cells [1, 2], which also produced 10-oxo-19-nor-25-hydroxycholecalciferol (10-oxo-19-nor-25-OHD_3). Synthesis of 8,25-$(OH)_2$-3-oxoneo-D_3 in phagocytic cells differed from that of 10-oxo-19-nor-25-OHD_3 in that 8,25-$(OH)_2$-3-oxoneo-D_3 was produced at longer incubation periods (12–24 h as compared with 1 h) and was not produced in the absence of phagocytic cells (nonenzymatically). It was proposed that a dioxygenase catalyzes 8,25-$(OH)_2$-3-oxoneo-D_3 synthesis in phagocytic cells [2].

The present authors subsequently demonstrated the conversion of 25-OHD_3 to 8,25-$(OH)_2$-3-oxoneo-D_3 by liver microsomes [3]. The liver is known to be the major site of production of 25-OHD_3 from cholecalciferol, but had not been shown previously to be a site of subsequent metabolism of 25-OHD_3. In this report, the characteristics of the reaction producing 8,25-$(OH)_2$-3-oxoneo-D_3 in liver microsomes are addressed and a function for this metabolite is proposed.

MATERIALS AND METHODS

Materials

Crystalline 24,25-dihydroxycholecalciferol [24,25-$(OH)_2D_3$], 25,26-dihydroxycholecalcif-

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Abbreviations: 25-OHD_3, 25-hydroxycholecalciferol; 1,25-$(OH)_2D_3$, 1,25-dihydroxycholecalciferol; 25,26-$(OH)_2D_3$, 25-26-dihydroxycholecalciferol; 24,25-$(OH)_2D_3$, 24,25-dihydroxycholecalciferol; 8,25-$(OH)_2$-3-oxoneo-D_3, 8α,25-dihydroxy-3-oxoneocholecalciferol, 8α,25-dihydroxy-9,10-seco-4,6,10(19)-cholestatrien-3-one; DPPD, *N,N'*-diphenyl-*p*-phenylenediamine.

erol [25,26-$(OH)_2D_3$] and 1,25-dihydroxycholecalciferol [1,25-$(OH)_2D_3$] were gifts from Dr Milan R. Uskokovic, Hoffmann-LaRoche, Nutley, NJ. Crystalline 25-OHD_3 was a gift from Dr John Babcock (Upjohn Co., Kalamazoo, MI). Concentrations of the cholecalciferol metabolites were determined in ethanol by u.v. absorbance (Lambda 3B u.v./vis spectrophotometer; Perkin–Elmer, Norvalk, CT) at 265 nm using a "molar" extinction coefficient of 18,200 $M^{-1}cm^{-1}$. Organic solvents were analytical or HPLC grade. Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, ATP, nicotinamide, $NADP^+$, ketoconazole, EDTA, progesterone, pregnenolone and *N,N'*-diphenyl-*p*-phenylenediamine (DPPD) were obtained from Sigma (St Louis, MO). 25-Hydroxy-[26,27-*methyl*-^{3}H]cholecalciferol was purchased from Amersham Corp. (Arlington Heights, IL) and was purified [4] before use on a 0.46 × 25 cm Zorbax-Sil HPLC column (Dupont Co., Wilmington, DE) with hexane–isopropanol (94:6, v/v) at a flow rate of 1 ml/min (Dupont 8800 Pump). Synthetic 25-OHD_3 served as standard.

Animals

Male Sprague–Dawley rats (Charles River Labs, Wilmington, MA) were maintained on a vitamin D-deficient diet [5] containing calcium and phosphorus at a level of 0.4% (ICN, Cleveland, OH). At sacrifice, rats were anesthetized with ether, guillotined and their livers excised.

Preparation of microsomes

The livers were rinsed with ice-cold 0.25 M sucrose and homogenized in 3 vol of 0.25 M sucrose, 0.05 M imidazole (pH 7.4) with a motor-driven glass–Teflon Potter–Elvehjem homogenizer. Postmitochondrial supernatant fractions were prepared from the livers by centrifugation at 12,000 *g* for 15 min at 4°C [6–8]. Microsomes (pellet) and cytosol (supernatant) were prepared from the 12,000 *g* supernatant by centrifugation at 105,000 *g* for 60 min at 4°C in an L8-70 ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA). The microsomal pellets were surface-rinsed twice with 0.25 M sucrose and resuspended in a volume of 0.25 M sucrose, 0.05 M imidazole equal to the volume of the postmitochondrial supernatant from which they were derived. The fluffy white layer at the top of the cytosolic fraction was removed with a Pasteur pipette and the remaining supernatant was used.

Incubation conditions

Microsomal suspensions (1.0 ml) were incubated in the presence of 2.0 mM magnesium acetate, 25 mM potassium acetate, 0.4 mM $NADP^+$, 20 mM nicotinamide, 4.0 mM glucose 6-phosphate, 1.2 U glucose 6-phosphate dehydrogenase, 12.8 μM DPPD, 33 mM imidazole (pH 7.4) and 1 ml of cytosol. Total incubation volume was 3.0 ml. Triplicate incubations were made. Each flask was gassed with oxygen for 1 min, after which [^{3}H]25-OHD_3 (1.7 μM, 60,000 dpm/nmol) or unlabeled 25-OHD_3 (25–83 μM) was added in 20 μl ethanol. The flasks were stoppered and incubated in a Dubnoff shaking incubator for 30 min at 37°C. Each incubation flask contained 5.0 mg microsomal protein and 18.2 mg cytosolic protein. In some experiments, cytosol was replaced by 0.25 M sucrose–0.05 M imidazole buffer. In each experiment, boiled microsomes, incubated in the same manner as unboiled microsomes, served as controls. The reactions were terminated by the addition of 1 vol of cyclohexane–ethyl acetate (1:1, v/v). Postmitochondrial supernatant (1.0 ml) was used in those incubations testing the effects of various agents. In these experiments, higher concentrations of unlabeled 25-OHD_3 (25–83 μM) were used, since it was found (unpublished results) that production of 8,25-$(OH)_2$-3-oxoneo-D_3 varied with concentration of 25-OHD_3 and had not yet reached saturation at 17 μM. In the incubations where NADH replaced $NADP^+$, other components of the NADPH-generating system (nicotinamide, glucose 6-phosphate and glucose 6-phosphate dehydrogenase) were included in the incubation mixture. Ketoconazole, DPPD, progesterone and pregnenolone were added to each incubation in 10 μl of ethanol. An equal volume of ethanol was added to control samples. EDTA was added in deionized water.

The incubation mixtures were extracted once with 2 vol [9] of cyclohexane–ethyl acetate (1:1, v/v) and once with 3 vol of ethyl ether (passed first over an alumina column). The extracts were combined and evaporated under nitrogen.

Chromatographic analysis

The lipid extracts were chromatographed on aluminum-backed silica gel 60 F_{254} TLC plates (E. Merck, Darmstadt, Germany). Lipid extracts of the incubation mixture were spotted under a stream of nitrogen as bands 2 cm wide.

Five samples were spotted 1 cm apart on a 20 × 20 cm TLC plate. Synthetic 24,25-$(OH)_2D_3$, 25,26-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$, spotted in the center of each plate, served as external standards. The plates were developed with chloroform–ethyl acetate (1:1, v/v) for 90 min [10, 11]. The migration distance was 14 cm. The standards were visualized under an u.v. lamp at 254 nm.

The regions on each TLC chromatogram that corresponded to the dihydroxy metabolites were scraped into 9 in. Pasteur pipets plugged with glass wool. The metabolite was eluted from the silica with 30 ml ethyl acetate. The ethyl acetate extracts of the dihydroxy metabolite regions were evaporated under nitrogen, redissolved in hexane–isopropanol (90:10, v/v), and chromatographed on a 0.46 × 25 cm Zorbax-Sil HPLC column (Dupont) with hexane–isopropanol (90:10, v/v) at a flow rate of 1 ml/min [4]. Synthetic 24,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$ served as standards. Ultraviolet absorption of the standards was monitored at 265 nm with a Waters Lambda-Max Model 481 liquid chromatography spectrophotometer (Waters Chromatography Division, Milford, MA). Radioactivity in the experimental samples was monitored by collecting (using a 2070 Ultrorac II fraction collector from LKB Produkter AB, Bromma, Sweden) and counting 30 1-ml fractions from the column. The fractions were evaporated, dissolved in 3a70B scintillation fluid (Research Products International Corp., Mt Prospect, IL), and counted in a Model LS 5801 liquid scintillation counter (Beckman Instruments Inc.).

The remainder of each of the TLC chromatograms were sectioned into three parts (from origin to front). The sections were cut into 20 ml counting vials and counted using 10 ml of 3a70B scintillation fluid. Disintegrations/min (dpm) were calculated for all samples counted using a stored standard curve (external standardization). The radioactivity found on the remainder of the TLC chromatogram was added to that obtained on each HPLC chromatogram in order to compute recovery. The average overall recovery was 57%. The radioactivity in the metabolite peak was corrected for recovery and the specific activity of the incubated 25-hydroxycholecalciferol (60,000 dpm/nmol) was used to calculate the number of nmol of metabolite formed. Boiled microsomes were incubated and evaluated in the same manner as the experimental samples. The metabolite was not present in the samples incubated with boiled microsomes.

In some experiments, samples were rechromatographed on a 0.46 × 25 cm Zorbax-Sil HPLC column (Dupont) with dichloromethane–isopropanol (95:5, v/v) at a flow rate of 1 ml/min and/or with dichloromethane–methanol (98:2, v/v) at a flow rate of 2 ml/min [12]. Synthetic 25-OHD_3, 24,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$ served as external standards. Chromatograms showing migration of 8,25-$(OH)_2$-3-oxoneo-D_3 in these three systems are available in an earlier publication [3] of the authors.

In those experiments employing nonlabeled 25-OHD_3, biosynthetic 8,25-$(OH)_2$-3-oxoneo-D_3 was employed as standard. An extinction coefficient of 21,878 M^{-1} cm^{-1} and the absorbance at 295 nm were used to determine the amount of 8,25-$(OH)_2$-3-oxoneo-D_3 chromatographed. A response factor was calculated by correlating the peak area of synthetic 8,25-$(OH)_2$-oxoneo-D_3 with the known amount of the synthetic standard chromatographed. This response factor was used to calculate the amount of 8,25-$(OH)_2$-oxoneo-D_3 produced in each incubation. Radioactive 25-OHD_3 (5000 dpm) was added to each incubation immediately following incubation. The percentage of radioactive 25-OHD_3 recovered was used to assess whether recovery was equivalent in all samples. Synthetic 25-OHD_3, monitored at 265 nm, served as a standard for the elution time of the radioactive tracer.

Triplicate incubations were made. The probability of difference between two means was determined using a two-tailed *t*-test. Proteins were assayed by the method of Lowry *et al.* [13] using bovine serum albumin as a standard.

1,25-Dihydroxycholecalciferol radioreceptor assay

Biosynthetic 8,25-$(OH)_2$-oxoneo-D_3 was purified and quantitated [3] for use in an assay of receptor binding. The radioreceptor assay kit (INCSTAR Corp., Stillwater, MN) contained the thymus receptor, which is specific for both 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_2$ [14]. The method employed was a nonequilibrium competitive binding assay.

RESULTS

8,25-$(OH)_2$-Oxoneo-D_3 was originally shown to be produced by liver microsomes in the

presence of an NADPH-generating system and the cytosolic fraction [3]. When the NADPH-generating system was omitted from the incubation mixture, the amount of 8,25-$(OH)_2$-3-oxoneo-D_3 produced was reduced approx. 77%, from 2.12 ± 0.08 to 0.49 ± 0.11 nmol/g liver per h (Table 1). NADH was capable of substituting for NADPH. In the presence of NADH, approx. 0.5% of the substrate in the incubation mixture was converted to product in 30 min. The effect of the two cofactors on the reaction cannot be compared since NADH was in the reduced state at the start of the incubations, but $NADP^+$, present at the start of the incubations, had to be reduced to NADPH by glucose 6-phosphate dehydrogenase. The rate of production of 8,25-$(OH)_2$-3-oxoneo-D_3 varied with the amount of postmitochondrial supernatant used (Fig. 1). 8,25-$(OH)_2$-Oxoneo-D_3 was not produced when the postmitochondrial supernatant was boiled.

When microsomes were incubated in the absence of cytosol, an apparent greater production of 8,25-$(OH)_2$-3-oxoneo-D_3 occurred, as evidenced by the size of the peaks from samples chromatographed on a silica column developed with hexane–isopropanol. However, when the metabolite region was rechromatographed using a different solvent system (Fig. 2), a single peak was obtained from those incubations made in the presence of cytosol (Fig. 2B), but two peaks, neither of which co-migrated with the metabolite, were obtained from those incubations made in the absence of cytosol (Fig. 2A). When 25-OHD_3 was incubated with cytosol only (the absence of microsomes), 8,25-$(OH)_2$-3-oxoneo-D_3 was not produced. In the presence of cytosol, DPPD stimulated microsomal production of the 8-hydroxy-3-keto metabolite from 2.06 ± 0.13 to 3.15 ± 0.16 nmol/g liver per h (Table 2). In the same experiment 3 mM EDTA had no effect on the production of the metabolite.

Table 1. The effect of NAD(P)H on production of 8,25-$(OH)_2$-oxoneo-D_3 by liver microsomes

Incubation conditions	8,25-$(OH)_2$-3-Oxoneo-D_3 production (nmol/g liver per h)
Postmitochondrial supernatant	0.49 ± 0.11
+NADPH generating system	2.12 ± 0.08
+1.0 mM NADH	2.92 ± 0.06
Boiled postmitochondrial supernatant	
+NADPH generating system	0
+1.0 mM NADH	0

Postmitochondrial supernatant from 0.25 g liver was incubated with potassium acetate, magnesium acetate, DPPD, imidazole buffer (pH 7.4) and 25 μM 25-OHD_3 for 30 min at 37°C. Product was analyzed as in Materials and Methods. Values are mean ± SD for 3 incubations per group.

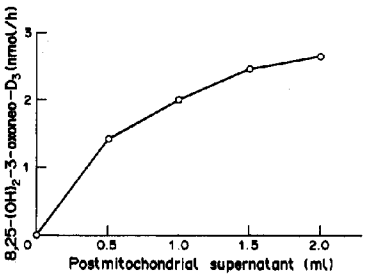

Fig. 1. Dependence of the production of 8,25-$(OH)_2$-3-oxoneo-D_3 on postmitochondrial supernatant content. Varying volumes of postmitochondrial supernatant were incubated with 17 μM 25-OHD_3 for 30 min at 30°C. Lipid extracts of the incubation mixtures were purified by TLC and by three different HPLC systems. Chromatograms showing migration of 8,25-$(OH)_2$-3-oxoneo-D_3 in these three systems are available in an earlier publication [3]. Production of 8,25-$(OH)_2$-3-oxoneo-D_3 was monitored by u.v. absorbance at 295 nm.

Production of 8,25-$(OH)_2$-3-oxoneo-D_3 was reduced by 34% in the presence of 50 μM ketoconazole (Table 3). Addition of progesterone and pregnenolone, at concentrations of 50 μM, reduced production of 8,25-$(OH)_2$-3-oxoneo-D_3 by 65 and 28%, respectively (Table 3).

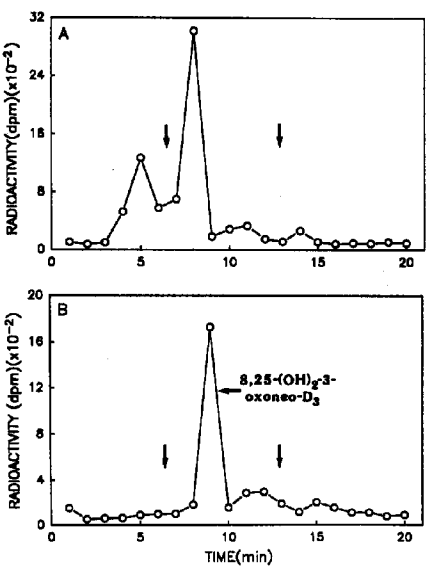

Fig. 2. Rechromatography of the radioactivity that co-eluted with 8,25-$(OH)_2$-3-oxoneo-D_3 on a Zorbax-Sil column developed with hexane–isopropanol (90:10, v/v) when microsomes were incubated in the absence (A) and presence (B) of cytosol. Rechromatography was on a Zorbax-Sil column developed with dichloromethane–methanol (95:5, v/v). The unlabeled arrows indicate the elution times of 25-OHD_3 and 24,25-$(OH)_2D_3$.

Table 2. Effect of antioxidants on the production of 8,25-$(OH)_2$-oxoneo-D_3 by liver microsomes

Incubation conditions	8,25-$(OH)_2$-3-Oxoneo-D_3 production (nmol/g liver per h)
Postmitochondrial supernatant	2.06 ± 0.13
+10 μM DPPD	3.15 ± 0.16
+3 mM EDTA	2.27 ± 0.05

Postmitochondrial supernatant from 0.25 g liver was incubated with 25 μM 25-OHD_3 in the presence of potassium acetate, magnesium acetate, imidazole buffer (pH 7.4) and an NADPH-generating system for 30 min at 37°C. Product was analyzed as in Materials and Methods. Values are mean ± SD for 3 incubations per group.

Table 3. Effect of various inhibitors on synthesis of 8,25-$(OH)_2$-oxoneo-D_3 by liver microsomes

Incubation conditions	8,25-$(OH)_2$-3-Oxoneo-D_3 production	
	nmol/g liver per h	% of control
Control	5.67 ± 0.46	100
+50 μM ketoconazole	3.76 ± 0.08	66
+50 μM pregnenolone	4.07 ± 0.30	72
+50 μM progesterone	2.01 ± 0.48	35

Postmitochondrial supernatant from 0.25 g liver was incubated in the presence of potassium acetate, magnesium acetate, DPPD, imidazole (pH 7.4), an NADPH-generating system and 42 μM 25-OHD_3 for 30 min at 37°C. Ketoconazole, pregnenolone and progesterone were added in ethanol. Values are mean ± SD for 3 incubations per group.

In a test of binding of 8,25-$(OH)_2$-oxoneo-D_3 to the 1,25-$(OH)_2D_3$ thymus receptor, 8,25-$(OH)_2$-3-oxoneo-D_3 failed to bind the receptor at concentrations 10-fold greater than that of 1,25-$(OH)_2D_3$ (Fig. 3). 25-OHD_3, the precursor of 1,25-$(OH)_2D_3$, has been shown to bind the thymus receptor at levels 10-fold greater than that of 1,25-$(OH)_2D_3$ [14].

DISCUSSION

8,25-$(OH)_2$-Oxoneo-D_3 was first discovered as a product of metabolism of 25-OHD_3 by phagocytic cells [1, 2]. A dioxygenase was suggested as the enzyme catalyzing the reaction and a mechanism for the oxidation was proposed [2]. The requirement for NAD(P)H in the production of 8,25-$(OH)_2$-3-oxoneo-D_3 by liver microsomes would suggest that the enzyme involved in formation of 8,25-$(OH)_2$-3-oxoneo-D_3 requires a second substrate and is, thus, not a dioxygenase. Although two sites of the 25-OHD_3 molecule are oxidized, two oxygens are not incorporated into the 25-OHD_3 substrate, a result expected if a dioxygenase were involved.

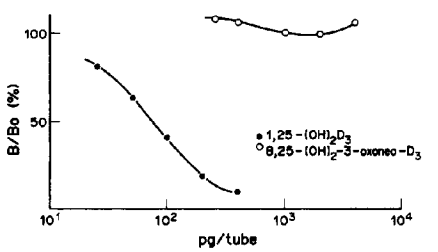

Fig. 3. Lack of affinity of 8,25-$(OH)_2$-3-oxoneo-D_3 for the thymus receptor for 1,25-$(OH)_2D_3$. A radioreceptor assay kit marketed by INCSTAR Corp. was used.

In this study, 25-OHD_3 was converted to 8,25-$(OH)_2$-3-oxoneo-D_3 in the presence of cytosol, but to two different unidentified peaks in the absence of cytosol. It is not known whether these two unidentified peaks are specific enzyme products or nonspecific oxidation products. These two peaks were produced in the presence of DPPD, an antioxidant. 8,25-$(OH)_2$-3-Oxoneo-D_3 has been shown previously to be the only significant metabolite of 25-OHD_3 that is biologically produced by microsomes in the presence of cytosol [3]. The production of 8,25-$(OH)_2$-3-oxoneo-D_3 in the presence of cytosol was stimulated by DPPD. The metal chelator (EDTA) neither stimulated nor inhibited the reaction. These results also suggest that 8,25-$(OH)_2$-3-oxoneo-D_3 is biologically produced. It appears that both DPPD and cytosol are necessary for optimal production of 8,25-$(OH)_2$-oxoneo-D_3 by liver microsomes.

The necessity for DPPD and cytosol when investigating cholecalciferol metabolism by liver microsomes has been established previously [6–8]. Bhattacharyya and DeLuca [6] have shown that degradation of cholecalciferol by liver microsomes was prevented by the addition of DPPD, but that maximum 25-hydroxylation of cholecalciferol was observed only when cytosol was added to the incubation. Cytosol both prevented degradation of cholecalciferol and stimulated 25-hydroxylase activity [6, 7]. In experiments by the authors [8], the cytosolic fraction stimulated the microsomal cholecalciferol 25-hydroxylase reaction in adult rats up to 2-fold. These data indicate that a cytosolic factor is also stimulatory for metabolism of 25-OHD_3 to 8,25-$(OH)_2$-3-oxoneo-D_3 by liver microsomes.

Hollis *et al.* [15] have shown recently that a M_w < 10,000 cytosol filtrate as well as a M_w < 1000 filtrate were capable of increasing 1,25-$(OH)_2D_3$ production by human trophoblastic mitochondria and microsomes up to 10-fold. The M_w < 10,000 filtrate had no effect on 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ production by pig kidney mitochondria, but stimulated 1,25-$(OH)_2D_3$ production by pig kidney microsomes 3-fold. The authors suggested that the production of 1,25-$(OH)_2D_3$ by microsomes and trophoblastic mitochondria involves the

insertion of oxygen at the 1 position of 25-OHD_3 by a free radical mechanism [15]. 1-Hydroxylation by these subcellular fractions was inhibited by DPPD and by EDTA. The data of Hollis for placental *in vitro* metabolism of 25-OHD_3 differ markedly from that for hepatic metabolism of 25-OHD_3. Present experiments suggest that the cytosolic fraction of liver stimulates conversion of 25-OHD_3 to 8,25-$(OH)_2$-3-oxoneo-D_3 and that this conversion is not inhibited by DPPD or EDTA. In these experiments, liver microsomes did not convert 25-OHD_3 to 1,25-$(OH)_2D_3$, either in the presence or absence of cytosol.

The protective and stimulatory effects of rat liver cytosol may be due to substrate stabilization [8]. For example, an α-tocopherol cytosolic binding protein specific to liver has been isolated and shown to transfer α-tocopherol from liposomes to microsomes [16]. Miller and Ghazarian [17] have isolated two binding proteins from chick liver cytosol, one that binds vitamin D_3 and 25-OHD_3 and a second that is specific for 25-OHD_3. Preliminary experiments by the authors indicate binding affinity for both vitamin D_3 and 25-hydroxyvitamin D_3 by a fraction of rat liver cytosol with $M_w > 100{,}000$ (unpublished results). The molecular weight of this fraction suggests that this protein is not identical to the 1,25-$(OH)_2D_3$ receptor or plasma vitamin D binding protein (DBP). A binding protein in this fraction might be involved in substrate or product stabilization. It is not known at this time whether this high molecular weight protein is a single polypeptide, a multimer or a complex.

Ketoconazole, a potent cytochrome *P*450 inhibitor, inhibited synthesis of 8,25-$(OH)_2$-3-oxoneo-D_3 34% at 50 μM. Steroid 16-β-hydroxylase activity, however, is reduced significantly (50%) by concentrations of ketoconazole as low as 0.12 μM [18]. Sheets *et al.* [18] reported that this selective inhibition is due to the direct interaction of imidazole antimycotics with the various forms of cytochromes *P*450. There exist multiple isozymes in liver microsomes, each with substantially different affinities for imidazole antimycotics [19]. Ketoconazole undoubtedly binds more specifically with the cytochrome *P*450 responsible for hydroxylation at the 16 position than it does to the isozyme responsible for 8,25-$(OH)_2$-3-oxoneo-D_3 production.

Since synthesis of 8,25-$(OH)_2$-3-oxoneo-D_3 involves formation of a ketone at C-3, the authors hypothesized that a 3β-hydroxysteroid dehydrogenase was involved in the synthesis of the metabolite. It had been reported recently that human liver microsomes possess the ability to convert 3α- and 3β-hydroxydesogestrel, a progestogen oral contraceptive, to the biologically active 3-keto form [20]. Inhibition studies suggested that the enzymes catalyzing the oxidation are possibly 3α- and 3β-hydroxysteroid dehydrogenases [20]. Pregnenolone, a substrate for the 3β-hydroxysteroid dehydrogenase–isomerase in the adrenals, inhibited synthesis of 8,25-$(OH)_2$-3-oxoneo-D_3 by 28%. Progesterone, the product of the 3β-hydroxysteroid dehydrogenase–isomerase reaction, inhibited synthesis by 65%. The two inhibitors were present in the incubation mixture at concentrations 1.2-fold greater than 25-OHD_3. It is not known whether pregnenolone and progesterone were metabolized by rat liver microsomes, because the chromatographic techniques employed were designed for the separation of cholecalciferol hydroxylated metabolites. These studies suggest, however, that the enzymes involved in the formation of this cholecalciferol metabolite do bind pregnenolone and progesterone.

It is proposed here that the enzymes that catalyze synthesis of 8,25-$(OH)_2$-3-oxoneo-D_3 are constitutively expressed in the liver and are able to bind and metabolize 25-OHD_3 to 8,25-$(OH)_2$-3-oxoneo-D_3. 8,25-$(OH)_2$-3-Oxoneo-D_3 is produced by liver microsomes from both male and female rats and from both cholecalciferol-deficient rats and cholecalciferol-replete rats (unpublished data). The substrate concentrations required for production of 8,25-$(OH)_2$-3-oxoneo-D_3 are relatively high compared with that required for microsomal cholecalciferol 25-hydroxylation in liver [7]. 8,25-$(OH)_2$-3-Oxoneo-D_3 was also unable to bind the thymus receptor for 1,25-$(OH)_2D_3$ at concentrations 10-fold greater than that of 1,25-$(OH)_2D_3$. Formation of 8,25-$(OH)_2$-3-oxoneo-D_3 at high substrate concentrations and the low affinity of 8,25-$(OH)_2$-3-oxoneo-D_3 for the thymus receptor suggest that this metabolite may be a degradative product of 25-OHD_3, which is formed when 25-OHD_3 levels in the liver are excessive. Synthesis of this metabolite in the liver may be catalyzed by enzymes that also metabolize other steroids.

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