Assay and Properties of Rat Yolk Sac 25-Hydroxyvitamin D_3 1α-Hydroxylase†

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ABSTRACT: An in vitro assay has been developed for the rat yolk sac 25-hydroxyvitamin D_3 1α-hydroxylase (1α-hydroxylase). The subcellular location and some properties of the enzyme are described. 1,25-Dihydroxyvitamin D_3 produced from incubations of yolk sac homogenates was extracted, purified by Sephadex LH-20 chromatography and straight- and reverse-phase high-performance liquid chromatography (HPLC), and measured by a competitive binding assay using chick intestinal receptor. The reaction is linear with time for up to 45 min at a substrate concentration of 80 μM and 4–6 mg/mL microsomal protein. The enzyme, located in the microsomes, requires molecular oxygen and NADPH. Metyrapone (1×10^{-3} M) was found to inhibit 1-hydroxylation, but a 90% carbon monoxide–10% oxygen atmosphere did not, leaving open the question of involvement of cytochrome P-450. Diphenyl-*p*-phenylenediamine, a lipid peroxidase inhibitor, inhibited 1α-hydroxylation.

It is well accepted that 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] is the hormonal form of vitamin D_3 active in bone calcium mobilization and intestinal calcium transport (DeLuca, 1980). The kidney was established as the in vivo site of 1,25-$(OH)_2D_3$ synthesis when Fraser and Kodicek (1970) showed that anephric rats were unable to convert vitamin D_3 to a more polar metabolite, later identified as 1,25-$(OH)_2D_3$ (Holick et al., 1971). This result has been confirmed (Gray et al., 1971). Recent studies have demonstrated that a variety of cell cultures are able to biosynthesize 1,25-$(OH)_2D_3$ (Howard et al., 1981; Puzas et al., 1980, 1983; Turner et al., 1980). However, the in vivo significance of these extrarenal hydroxylations is in serious doubt since no 1,25-$(OH)_2D_3$ can be detected in anephric animals (Reeve et al., 1983; Shultz et al., 1983).

Pregnancy is associated with changes in the vitamin D endocrine system. Among these changes is an increase in the maternal circulating levels of 1,25-$(OH)_2D_3$ in the last trimester of pregnancy in both man and laboratory animals (Gray et al., 1981). Anephric pregnant rats are able to produce a compound believed to be 1,25-$(OH)_2D_3$ (Lester et al., 1978; Weisman et al., 1978). One author provided evidence that the 1,25-$(OH)_2D_3$ produced in vivo by anephric, pregnant animals was of placental origin (Lester et al., 1978). Subsequently, Tanaka et al. (1979) demonstrated that rat placental homogenates are able to produce 1,25-$(OH)_2D_3$. These authors identified the product 1,25-$(OH)_2D_3$ by cochromatography on three different chromatographic systems (Sephadex LH-20 chromatography and straight- and reverse-phase HPLC), ultraviolet absorption spectrophotometry, and mass spectrometry. Thus, placenta must be regarded as an active in vivo site of 1,25-$(OH)_2D_3$ synthesis.

The renal 1α-hydroxylase has been well studied in the chick (Ghazarian & DeLuca, 1974; Henry & Norman, 1974; Pedersen et al., 1976). The enzyme appears to be a mitochondrial cytochrome P-450 containing monooxygenase similar to adrenal steroidgenic hydroxylases. The nature of the enyzme in rat placenta that catalyzes the 1-hydroxylation of 25-hydroxyvitamin D_3 (25-OH-D_3) is not known. A recent study found the human placenta 1α-hydroxylase to be of mitochondrial origin (Zerwekh & Breslau, 1986). A 25-OH-D_3 24-hydroxylase has been shown to be present in rat yolk sac (Danan et al., 1982). The yolk sac, a part of the placenta, is of fetal origin and is active in the transfer of nutrients during the early stages of gestation (Ramsey, 1975). The yolk sac 24-hydroxylase was found to be located in the mitochondria and to be sensitive to metyrapone (Danan et al., 1982). In this study, a 25-OH-D_3 1α-hydroxylase was also found in the rat yolk sac but is found in the microsomes. This paper reports these findings and other properties of this system.

Materials and Methods

Vitamin D Compounds. The 1,25-$(OH)_2$[26,27-^{3}H]D_3 from New England Nuclear Corp. (Boston, MA) had a specific activity of 160 Ci/mmol and was synthesized by previously published methods (Napoli et al., 1979). The 25-OH-D_3 was kindly donated by Drs. Babcock and Campbell of the Upjohn Co. (Kalamazoo, MI).

Animals. Pregnant rats at 16–19 days of gestation were purchased from the Holtzman Co. (Madison, WI) and placed on a vitamin D deficient (0.47% calcium/0.3% phosphorus) diet for 1–2 days until sacrifice. The animals were housed in individual wire cages and allowed food and water ad libitum.

Preparation of Microsomes. Animals were killed by decapitation. Yolk sacs were separated from fetus, uterus, and chorioallantoic placenta and placed immediately into ice cold 0.19 M sucrose. The tissue was minced, washed several times, and filtered through two layers of cheesecloth. A 10% yolk sac homogenate was prepared in 0.19 M sucrose. The homogenate was centrifuged at 150*g* at 4 °C for 20 min. The supernatant was centrifuged at 4300*g* at 4 °C for 20 min to remove mitochondria. To separate microsomes from the soluble fraction, the supernatant was centrifuged at 100000*g* for 60 min at 4 °C. The microsomal pellet was resuspended in 0.19 M sucrose to a protein concentration of 4–6 mg/mL. Protein was determined by the method of Bradford (1976).

Subcellular Fractionation. All fractions were prepared as described above, except the nuclei and mitochondrial pellets were washed twice and once, respectively. Succinate dehydrogenase and NADH-cytochrome *c* reductase (Green et al., 1955; Schneider & Hogeboom, 1952) were used as marker

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enzymes to determine the purity of the mitochondrial and microsomal fractions. All fractions were resuspended in 0.19 M sucrose to the original volume.

Whole Placenta Fractionation. Pregnant animals were sacrificed and dissected as described above. Whole rat placentae were separated into two anatomical parts: choriovitteline or yolk sac placenta and chorioallantoic placenta. The yolk sac is a bilaminar structure that surrounds the fetus and is easily distinguished from the dish-like chorioallantoic placenta. The yolk sac is connected to the fetus by the vitteline duct (Ramsey, 1975). A homogenate of each tissue was prepared in 0.19 M sucrose. The following tissue amounts in 2 mL of 0.19 M sucrose were assayed for 1α-hydroxylase activity as described: (1) 100 mg of choriovitteline placenta, (2) 100 mg of chorioallantoic placenta, and (3) 100 mg of choriovitteline placenta and 100 mg of chorioallantoic placenta.

Incubations and Extractions. Two milliliters of microsomal, tissue, or subcellular fraction suspension was placed in a 125-mL Erlenmeyer flask along with 0.5 mL of phosphate buffer, pH 7.4, containing 33.3 mM glucose 6-phosphate, 30 mM adenosine triphosphate, 240 mM nicotinamide, and 2.4 mM NaDP. A 0.5-mL salt solution containing 12 mM magnesium acetate, 0.5 M potassium acetate, and 0.5 unit of glucose-6-phosphate dehydrogenase (Sigma Co., St. Louis, MO) was also added to the flask. Incubations were at 37 °C for 20 min unless otherwise indicated. Reactions were initiated by the addition of 200 μg of 25-OH-D_3 in 25 μL of 95% ethanol and terminated by the addition of 3 volumes of methanol/methylene chloride (2:1, v/v). Samples were extracted according to the method of Shepard et al. (1979). Assays were performed in duplicate or triplicate. Recoveries were monitored by the addition of tracer 1,25-$(OH)_2[^3H]D_3$ (3000 cpm/flask) after termination of the reaction. One control was a flask to which no substrate was added, and another was a flask that contained boiled instead of fresh tissue. All experiments were repeated at least twice with identical results.

Purification and Measurements. The organic phase of the extract was evaporated to dryness with a rotary evaporator. The residue was dissolved in 500 μL of hexane–chloroform–methanol (9:1:1) and applied to a Sephadex LH-20 column (0.7 × 14 cm) suspended in the same solvent mixture (Shepard et al., 1979). The column was eluted with 25 mL of the same solvent, and the last 15 mL, which contained the 1,25-$(OH)_2D_3$ fraction, was collected and dried under a stream of nitrogen. The residue was dissolved in 300 μL of 10% 2-propanol in hexane and chromatographed by straight-phase HPLC on a Waters Model ALC/GPC 204 (Waters Associates, Milford, MA) HPLC apparatus equipped with a Du Pont Zorbax-Sil column (4.6 mm × 25 cm) eluted at a flow rate of 2 mL/min in 10% 2-propanol/90% hexane and at a pressure of 1100 psi. The region containing 1,25-$(OH)_2D_3$ was further purified by reverse-phase HPLC on a Du Pont Zorbax-ODS column (4.6 mm × 25 cm) at a flow rate of 2 mL/min in 20% water/80% methanol and 1800 psi. Elution positions were determined with authentic 1,25-$(OH)_2D_3$. 1,25-$(OH)_2D_3$ was determined by a competitive binding assay using the chick intestinal receptor for a binding protein (Eisman et al., 1976).

Identification of Rat Yolk Sac Microsomal 1,25-$(OH)_2D_3$. Yolk sacs were obtained from five pregnant rats at day 20 of gestation. Whole placenta fractionation and preparation of microsomes were as previously described in this paper. Incubation of yolk sac microsomes with 25-OH-D_3 was as indicated above except the components added to each flask were increased by a factor of 2.5 to produce enough 1,25-$(OH)_2D_3$ for identification. A total of five flasks was incubated for 1 h at 37 °C. The reaction was stopped by addition of 3 volumes of methanol–methylene chloride (2:1), and the contents of the flasks were combined. The sample was extracted and subjected to Sephadex LH-20 chromatography as previously stated. The fractions containing 1,25-$(OH)_2D_3$ were purified in succession by the following three procedures: (1) straight-phase HPLC with a Du Pont Zorbax-Sil column (4.6 mm × 25 cm) at a flow rate of 2 mL/min in 10% 2-propanol/90% hexane; (2) reverse-phase HPLC with a Du Pont Zorbax-ODS column (4.6 mm × 25 cm) at a flow rate of 2 mL/min in 20% water/80% methanol; (3) straight-phase HPLC with a Zorbax-Sil column (4.6 mm × 25 cm) at a flow rate of 2 mL/min in 5% 2-propanol/95% methylene chloride. In each of these chromatographic procedures, the fractions containing 1,25-$(OH)_2D_3$ were determined with tritiated hormone prior to the application of sample. Aliquots of the 1,25-$(OH)_2D_3$ elution region from the straight-phase HPLC step (5% 2-propanol/95% methylene chloride elution solvent) and reverse-phase HPLC step from a separate but identical experiments were tested for the ability to bind to the chicken intestinal 1,25-$(OH)_2D_3$ receptor.

Changes in Incubation Atmosphere. Microsomes, buffer, and all cofactors were placed in a 120-mL serum bottle on ice and sealed with a rubber serum stopper. The flasks were attached to vacuum and gas lines through needles placed into the stopper. To produce a 100% nitrogen or 100% oxygen atmosphere, flasks were evacuated and flushed with nitrogen 3 times, reevacuated, and then flushed with the appropriate gas. To produce a 90% carbon monoxide–10% oxygen atmosphere, or a 90% nitrogen–10% oxygen atmosphere, flasks were evacuated and flushed with nitrogen 3 times, reevacuated, and filled with 100% nitrogen or 100% carbon monoxide. The flasks were allowed to sit on ice for 20 min prior to the addition of the appropriate amount of oxygen. 25-OH-D_3 was also added with a syringe.

Scintillation Counting. Samples from HPLC (air-dried) and aqueous binding assay samples were counted in 2.5 mL of counting solution (Scint A, United Technologies Packard) by a Packard Model 400 CL/D liquid scintillation counter (Packard Instruments, Downers Grove, IL).

Results

Tissue Distribution of Rat Placenta 25-OH-D_3 1α-Hydroxylase. The yolk sac and chorioallantoic placenta represents two different anatomical parts of the rat whole placenta (Ramsey, 1975). Both the yolk sac and chorioallantoic placenta contained 1α-hydroxylase activity. The activity of the 1α-hydroxylase enzyme is highest in the yolk sac placenta [3550 pg $(20\ min)^{-1}$ $(100\ mg\ of\ tissue)^{-1}$], as compared with the chlorioallantoic placenta [1091 pg $(20\ min)^{-1}$ $(100\ mg\ of\ tissue)^{-1}$]. The 1$\alpha$-hydroxylase activity of the combined yolk sac and chorioallantoic placenta approximates the sum of the 1α-hydroxylase activity found when the two tissues were assayed separately [4754 pg $(20\ min)^{-1}$ (100 mg of yolk sac and 100 mg of chlorioallantoic placenta)$^{-1}$]. Subsequent studies defining the properties of the 1α-hydroxylase were carried out on yolk sac preparations.

Identification of 1,25-$(OH)_2D_3$ Produced by Rat Yolk Sac Microsomes. Rat yolk sac microsomes incubated with 25-OH-D_3 produced a compound, detected by UV absorbance at 254, that comigrated with authentic 1,25-$(OH)_2D_3$ on Sephadex LH-20 chromatography and three successive HPLC systems including (1) straight-phase HPLC (10% 2-propanol/90% hexane solvent system), (2) reverse-phase HPLC (20% water/80% methanol solvent system), and (3)

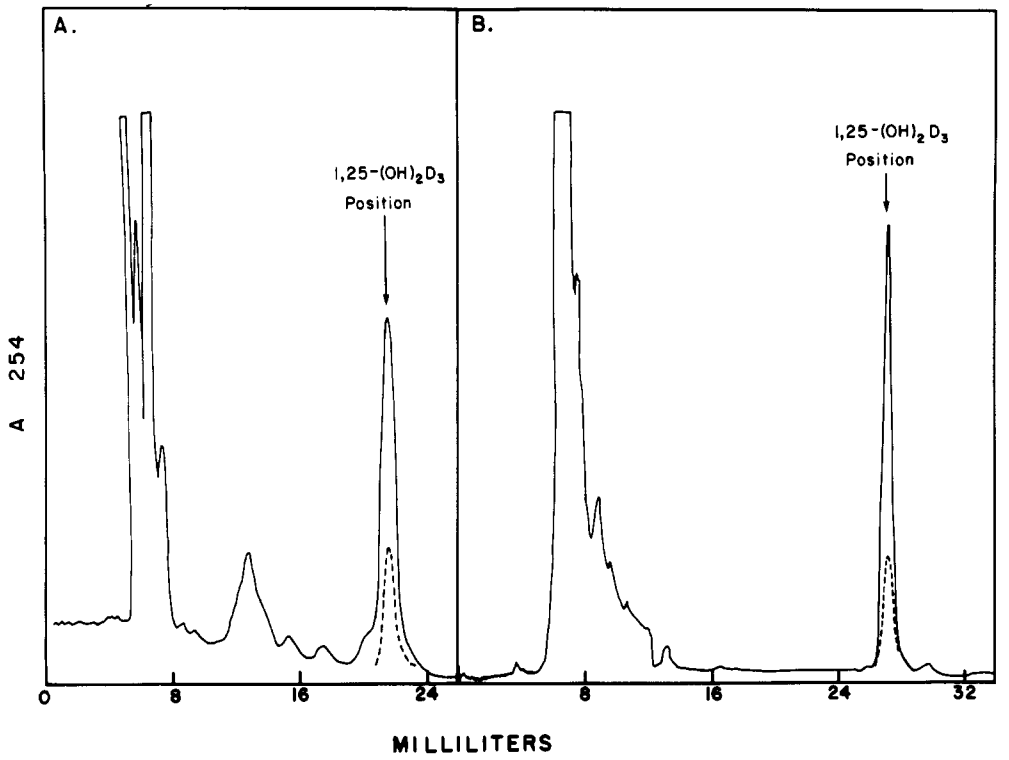

FIGURE 1: HPLC profile of putative 1,25-$(OH)_2D_3$ from straight-phase HPLC (Zorbax-Sil column, 10% 2-propanol–90% hexane solvent system) on (A) reverse-phase HPLC (Zorbax-ODS column, 20% H_2O–80% methanol) followed by (B) straight-phase HPLC (Zorbax-Sil column, 5% 2-propanol–95% methylene chloride). The dashed lines represent authentic 1,25-$(OH)_2D_3$, and the solid lines represent the OD of the sample.

Table I: Binding Affinity of Authentic 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ Produced by Rat Yolk Sac Microsomes to Chicken Intestinal 1,25-$(OH)_2D$ Receptor[a]

source of 1,25-$(OH)_2D_3$	HPLC peak height (cm)	1,25-$(OH)_2D_3$ mass estimated by binding assay (ng)
Straight-Phase HPLC		
authentic 1,25-$(OH)_2D_3$	17	154
yolk sac 1,25-$(OH)_2D_3$	17	136
Reverse-Phase HPLC		
authentic 1,25-$(OH)_2D_3$	9.5	91
yolk sac 1,25-$(OH)_2D_3$	9.5	79

[a] The sensitivity of the detector was at 0.01 milliabsorbance units. The reverse-phase and straight-phase HPLC samples were from separate but identical experiments.

straight-phase HPLC (5% 2-propanol/95% methylene chloride solvent system) (Figure 1). The fractions comigrating with authentic 1,25-$(OH)_2D_3$ from straight-phase HPLC (5% 2-propanol/95% methylene chloride solvent system) and reverse-phase HPLC had 88% and 86% of the binding affinity to the chicken intestinal 1,25-$(OH)_2D_3$ receptor as authentic 1,25-$(OH)_2D_3$ of the same peak area eluting from similar HPLC chromotograms (Table I). For routine assay of yolk sac microsomal 1α-hydroxylase, the straight-phase (10% 2-propanol/90% hexane solvent system) and reverse-phase HPLC steps were found to be adequate for purification of 1,25-$(OH)_2D_3$ as indicated by (1) a single peak on the straight-phase HPLC chromatogram (5% 2-propanol/95% methylene chloride) after application of the reverse-phase sample and (2) similar ratios of peak height to binding affinity of the samples from the reverse-phase HPLC and straight-phase HPLC (5% 2-propanol/95% methylene chloride).

Subcellular Distribution of Yolk Sac 25-OH-D_3 1α-Hydroxylase. The subcellular distribution of the yolk sac 1α-hydroxylase is shown in Table II. The highest total enzyme activity and the highest specific activity were located in the microsomal fraction. Ninety-four percent of the total protein and 70.6% of the total enzyme activity were recovered during fractionation. The purity of the microsomal fraction was determined with NADH-cytochrome *c* reductase, whose location in liver is split between mitochondria and microsomes, 35% and 65%, respectively (DeDuve et al., 1955). In our study we found yolk sac NADH-cytochrome *c* reductase to be lo-

Table II: Subcellular Distribution of Rat Yolk Sac 25-OH-D 1-Hydroxylase Activity[a]

fraction	sp act. [pg of 1,25-$(OH)_2D_3$ $(20\ min)^{-1}$ $(mg\ of\ protein)^{-1}$]	total enzyme act. (pg of 1,25-$(OH)_2D_3$/ 20 min)	total protein (mg)
whole homogenate	111.5	2524, 2026 (2275)	20.0, 20.8 (20.4)
nuclei	76	99, 21, 435 (183)	2.4, 2.4 (2.4)
mitochondria	84	354, 300, 389 (345)	4.2, 4.1 (4.1)
microsomes	177	1247, 1003, 697 (1080)	5.7, 6.6 (6.1)
cytosol	0	0, 0, 0 (0)	6.4, 6.0 (6.2)
% recovered		(70.6)	(94.0)

[a] Incubations were carried out as described under Materials and Methods, except the buffer solution contained 25 mM succinate, as well as the NADPH-generating system. Duplicate or triplicate values are shown. The mean values are given in parentheses.

Table III: Activity of NADH-Cytochrome *c* Reductase and Succinate Dehydrogenase in Subcellular Fractions of Rat Yolk Sac[a]

subcellular fraction	percent of total activity: NADH-cytochrome *c* reductase	percent of total activity: succinate dehydrogenase
whole homogenate	100	100
nuclei	6.2	52.7
mitochondria	21.7	51.6
microsomes	52.1	0
cytosol	1.0	0
activity recovered (%)	82.1	104.3

[a] Assay conditions are as described under Materials and Methods.

Table IV: Effects of NADPH and a NADPH-Generating System on Activity of Yolk Sac Microsomal 25-OH-D_3 1-Hydroxylase[a]

group	source of reducing equivalent	$1,25\text{-}(OH)_2D_3$ produced [pg (20 min)$^{-1}$ (mg of protein)$^{-1}$]
1	none	72, 61, 84
2	NADPH (0.25 M)	103, 76
3	NADPH (0.5 M)	74, 95
4	NADPH (1.0 M)	55, 122
5	NADPH-generating system	175, 173, 174

[a] Groups 1–4 were as described under Materials and Methods, except the components of the NADPH-generating system were not added and NADPH was added in the phosphate buffer to the incubation at the concentrations given above. Duplicate or triplicate values are shown.

cated in mitochondria and microsomes, 21% and 52%, respectively (Table III). Further evidence of microsomal location for the 1α-hydroxylase is the observation that in whole yolk sac homogenates an NADPH-generating system is able to support 1-hydroxylation [1319 pg of $1,25\text{-}(OH)_2D_3$ (20 min)$^{-1}$ (100 mg of tissue)$^{-1}$], while minimal support was provided by succinate [646 pg of $1,25\text{-}(OH)_2D_3$ (20 min)$^{-1}$ (100 mg of tissue)$^{-1}$ as compared to when no reducing equivalents were added [545 pg of $1,25\text{-}(OH)_2D_3$ (20 min)$^{-1}$ (100 mg of tissue)$^{-1}$]. All subsequent experiments were therefore performed with yolk sac microsomal preparations.

Kinetic Properties of Yolk Sac Microsomal 25-OH-D_3 1α-Hydroxylase. The time course and substrate concentration curves for the conversion of 25-OH-D_3 to $1,25\text{-}(OH)_2D_3$ in yolk sac microsomes are in Figure 2. The reaction is linear for up to 45 min and reached saturation with 80 μM substrate. The need for large amounts of substrate may indicate that, as previously observed with the rat kidney 1α-hydroxylase (Botham et al., 1974), the microsomal preparation is contaminated with the plasma vitamin D binding protein.

Requirements of Yolk Sac Microsomal 25-OH-D_3 1α-Hydroxylase. The effects of increasing concentrations of NADPH and a NADPH-generating system on 1α-hydroxylase activity in yolk sac microsomes are summarized in Table IV. NADPH added in the reduced form to the incubation mixture supported 1-hydroxylation only slightly better than a system without any added cofactors. An NADPH-generating system (glucose-6-phosphate dehydrogenase and glucose 6-phosphate and ATP) was the most efficient in providing reducing equivalents for the reaction.

Oxygen Requirement of Yolk Sac Microsomal 25-OH-D_3 1α-Hydroxylase. 1-Hydroxylation in the yolk sac was inhibited 93% when incubated in a 100% nitrogen atmosphere [6 ± 1.2 pg (20 min)$^{-1}$ (mg of protein)$^{-1}$, mean ± SE] instead of a 100% oxygen atmosphere [76 ± 3.7 pg (20 min)$^{-1}$ (mg of protein)$^{-1}$, mean ± SE]. The activity of the 1α-hydroxylase was lower in the 100% oxygen atmosphere [76 ± 3.7 pg (20 min)$^{-1}$ (mg of protein)$^{-1}$, mean ± SE] when compared to an air atmosphere [138 ± 31.5 pg (20 min)$^{-1}$ (mg of protein)$^{-1}$, mean ± SE].

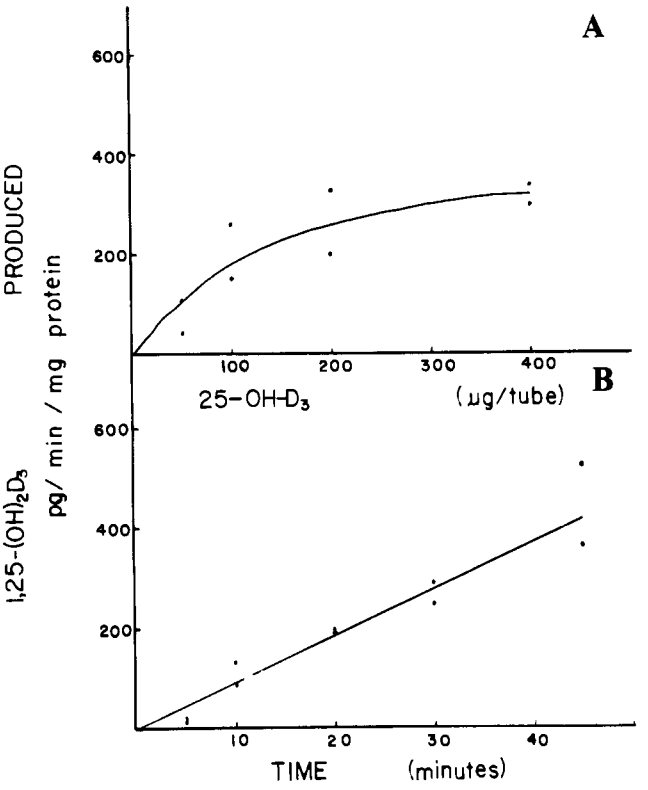

FIGURE 2: Effect of substrate concentration (A) and incubation time (B) on in vitro production of $1,25\text{-}(OH)_2D_3$ by placental microsomes.

Effect of Metabolic Inhibitors on Yolk Sac Microsomal 25-OH-D_3 1α-Hydroxylase. Metyrapone (1×10^{-3} M), a competitive inhibitor of substrate binding to P-450, decreased yolk sac 1α-hydroxylase activity from a control value of 138 ± 32 pg (20 min)$^{-1}$ (mg of protein)$^{-1}$, mean ± SE, to 85.4 ± 1.9 pg (20 min)$^{-1}$ (mg of protein)$^{-1}$, mean ± SE. However, carbon monoxide (90% carbon monoxide–10% oxygen atmosphere), another specific inhibitor of cytochrome P-450 enzymes, had no effect upon enzymatic activity [274 ± 57 pg of $1,25\text{-}(OH)_2D_3$ (20 min)$^{-1}$ (mg of protein)$^{-1}$, mean ± SE] when compared to enzyme activity from incubations in a 90% nitrogen–10% oxygen atmosphere [270 ± 27 pg of $1,25\text{-}(OH)_2D_3$ (20 min)$^{-1}$ (mg of protein)$^{-1}$, mean ± SE]. Diphenyl-*p*-phenylenediamine or DPPD (1×10^{-5} M), an antioxidant that typically inhibits lipid peroxidase reactions, completely inhibited 1-hydroxylase activity in the yolk sac to levels not detectable by our assay.

Discussion

The observation that bilateral nephrectomy of the pregnant rat does not completely prevent production of $1,25\text{-}(OH)_2D$ provided the first evidence that a placental 1-hydroxylase might exist (Gray et al., 1981; Weisman et al., 1978). Subsequently, it was demonstrated that rat placental tissue could produce a compound in vitro that was positively identified as $1,25\text{-}(OH)_2D_3$ (Tanaka et al., 1979). Reports of 1-hydroxylase activity in human decidua (Weisman et al., 1979) and villous tissue (Whitsett et al., 1981) soon followed. An assay for rat placental 1α-hydroxylase activity has been reported for serum-free isolated placental cells. With such a measurement, these investigators concluded that rat maternal placenta had a 1α-hydroxylase activity higher than that found in kidneys from both pregnant and nonpregnant female rats (Gray & Lester, 1981).

The present study demonstrates that rat yolk sac microsomes are capable of metabolizing 25-OH-D_3 to 1,25-$(OH)_2D_3$. The product, 1,25-$(OH)_2D_3$, in this study was identified with certainty by four different chromatographic systems that included Sephadex LH-20 chromatography and straight- and reverse-phase HPLC. Both the reverse-phase and straight-phase 5% 2-propanol/95% methylene chloride solvent systems employed in the present study are able to separate 19-nor-10-keto-25-hydroxyvitamin D_3 from 1,25-$(OH)_2D_3$ (Brown & DeLuca, 1985; Simpson et al., 1984; Napoli et al., 1983). 19-Nor-10-keto-25-hydroxyvitamin D_3 is a compound that comigrates with 1,25-$(OH)_2D_3$ on straight-phase HPLC with 10% 2-propanol/90% hexane solvent system and can interfere with measurement of 1,25-$(OH)_2D$ produced by in vitro systems. It has some activity in binding to the 1,25-$(OH)_2D_3$ receptor in chick intestine (Simpson et al., 1984). HPLC-purified 1,25-$(OH)_2D_3$ produced by yolk sac microsomes was found equally active as authentic 1,25-$(OH)_2D_3$ in displacing tritiated 1,25-$(OH)_2D_3$ from chick intestinal receptor, a property not attributable to any other vitamin D metabolite. Previously, investigators in this laboratory showed that homogenates of whole placenta were able to biosynthesize 1,25-$(OH)_2D_3$ using identical incubation and chromatographic conditions as in the present study (Tanaka et al., 1979). The 1,25-$(OH)_2D_3$ produced in that study using whole placenta homogenates was unequivocally identified by ultraviolet absorption spectrophotometry and mass spectrometry. Thus, there is no doubt that the product is, in fact, 1,25-$(OH)_2D_3$.

Similar to the findings of Tanaka and DeLuca (1981) with the rat kidney, we found that large amounts of substrate were necessary for the expression of yolk sac microsomal 1α-hydroxylase activity. The first attempts to measure rat kidney 1α-hydroxylase were unsuccessful due to the presence of an inhibitor. The inhibitor has been isolated and identified as the plasma vitamin D transport protein (Botham et al., 1974). This iron-containing protein, which contaminates tissue homogenates, binds the 25-OH-D with high affinity. To circumvent this inhibition in the kidney, an assay was developed that utilized excess 25-OH-D to saturate the plasma vitamin D binding protein and provide enough substrate for the 1α-hydroxylase. Because of the large substrate requirement for the microsomal yolk sac 1α-hydroxylase, it is likely that the plasma vitamin D binding protein is also an inhibitor of this enzyme. Indeed, the microsomal fraction of the rat kidney contains the highest inhibitor activity of the 1α-hydroxylase (Botham et al., 1974).

We found the yolk sac 1α-hydroxylase to be located in the microsomal fraction. This was confirmed by subcellular fractionation studies and the ability of an NADPH-generating system to support 1-hydroxylation in whole yolk sac homogenates better than succinate (Tables II and III). NADPH, which does not readily cross the mitochondrial membrane, is an inadequate supplier of reducing equivalents to mitochondrial enzymes and a good electron donor for microsomal enzymes. Succinate only supplies an intramitochondrial source of NADPH. In the subcellular fractionation studies, the marker enzymes succinate dehydrogenase and NADPH-cytochrome *c* reductase were employed to identify mitochondrial and microsomal fractions. Succinate dehydrogenase activity was found only in the mitochondrial and nuclear fractions, indicating that the microsomes were not contaminated with mitochondria (Table III). Small amounts of 1α-hydroxylase activity was found in the nuclei and mitochondria; however, the specific activity of the enzyme was the highest in the microsomes. The presence of the enzyme in the nuclei and mitochondria is very likely due to microsomal contamination (Table III). A recent study found whole human placenta 25-OH-D_3 1α-hydroxylase to be located in the mitochondria (Zerwekh & Breslau, 1986). This is in contrast to our findings in the rat. Whether this discrepancy represents a species difference has yet to be determined. In contrast to the yolk sac 1-hydroxylase, the chick (Gray et al., 1972) and rat (Paulson & DeLuca, 1985) kidney 1α-hydroxylases are located in the mitochondrial fraction of the cell. The significance of the different subcellular location for the rat yolk sac 1α-hydroxylase is not known.

Other investigators (Danan et al., 1982) were able to detect 25-OH-D and 1,25-$(OH)_2D$ 24-hydroxylase activity in rat yolk sac mitochondria with 1,25-$(OH)_2D$ being the preferential substrate. These data are interesting considering the present finding of a microsomal location for the yolk sac 1α-hydroxylase. In the former study it is not clear whether the investigators searched the microsomal fraction for the presence of the 24-hydroxylase or not. In the chick, the 25-OH-D 1α- and 24-hydroxylases are found in the mitochondria (Gray et al., 1972; Knutson & DeLuca, 1974).

The yolk sac 25-hydroxyvitamin D_3 1-hydroxylase possesses characteristics similar to mixed-function oxidases in that the enzyme requires molecular oxygen and NADPH for maximal activity (Cammer et al., 1968). In our studies, substantial product formation was obtained without added cofactors. It appears that cofactors necessary for 1α-hydroxylase are present in the yolk sac microsomes in amounts capable of supporting limited 1-hydroxylation. However, an NADPH-generating system was required for maximal activity. Maximal enzyme activity could not be obtained with the addition of NADPH alone. It is possible that other components of the NADPH-generating system such as ATP are necessary for maximal enzyme activity. Incubation in a 100% nitrogen atmosphere blocked 1-hydroxylation in this study by over 90%. This indicates that the source of oxygen for the hydroxyl group inserted into the 1-position of 25-OH-D_3 is molecular oxygen. The 100% oxygen atmosphere appears to inhibit 1α-hydroxylase activity when compared to the air atmosphere. The reason for this phenomenon is not known. It is possible that the 100% oxygen atmosphere is supporting oxygenation of the product, 1,25-$(OH)_2D_3$.

Whether or not the yolk sac microsomal 1α-hydroxylase is a cytochrome P-450 containing monooxygenase cannot be determined from this study. Metyrapone (1×10^{-3} M), a competitive inhibitor of substrate binding to P-450 enzymes, was found to inhibit yolk sac 1α-hydroxylase, while 90% carbon monoxide did not. The inhibition of yolk sac 1α-hydroxylation by DPPD, a free radical scavenger and characteristic inhibitor of lipid peroxidase reactions, indicates that the 1α-hydroxylase in yolk sac microsomes may not be a typical mixed-function oxidase. This can only be determined by solubilization and more detailed study of the enzyme.

However, this study shows that 1α-hydroxylation in rat yolk sac is not related to the three-component mixed-function monooxygenase found in renal mitochondria and that, in this case, it may be a more primitive microsomal lipid peroxidase system.

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