Evidence for Extra-Renal 1 α-Hydroxylation of 25-Hydroxyvitamin D_3 in Pregnancy

Abstract. *The kidneys are thought to be the only organs capable of 1 α-hydroxylation of vitamin D and its metabolites. We have examined the in vivo conversion of ^{3}H-(25,26)-25-hydroxyvitamin D_3 (25OHD$_3$) to ^{3}H-(25,26)-1 α,25-dihydroxyvitamin D_3 [1α,25(OH)$_2$D$_3$] in vitamin D-deficient, pregnant and nonpregnant rats. As expected, nephrectomy of nonpregnant, vitamin D-deficient rats prevented the conversion of 25OHD$_3$ to 1α,25(OH)$_2$D$_3$. In contrast, nephrectomy of pregnant, vitamin D-deficient rats reduced but did not abolish the formation of 1 α,25(OH)$_2$D$_3$ from its precursor. The identity of the radioactive metabolite formed from ^{3}H-25OHD$_3$ which circulated in nephrectomized, pregnant rats was established as 1α,25(OH)$_2$D$_3$ by comigration with synthetic 1 α,25(OH)$_2$D$_3$ on high-pressure liquid chromatography. The simultaneous absence of 1 α,25(OH)$_2$D$_3$ in the fetal kidneys indicated that the site of 1 α-hydroxylation after nephrectomy of the pregnant rat was probably extra-renal in origin. Two sites of 1 α-hydroxylation of 25OHD$_3$, one renal and the other extra-renal, either fetoplacental or maternal, may exist in the pregnant, vitamin D-deficient rat.*

The most biologically active metabolite of vitamin D is 1 α,25-dihydroxyvitamin D [1 α,25(OH)$_2$D$_3$] (*1*). A unique feature of the biosynthesis of 1 α,25(OH)$_2$D$_3$ is the fact that 1 α-hydroxylation of this metabolite is thought to occur exclusively in the kidneys (*1, 2*). Several lines of evidence have established the renal location of this metabolic step: (i) nephrectomy prevents the formation of 1 α, 25(OH)$_2$D$_3$ from its precursors and also blocks the responses of the small intestine and the skeleton, which are observed after the administration of 25-hydroxyvitamin D$_3$ (25OHD$_3$) to animals with intact kidneys (*3, 4*); (ii) the blood obtained from anephric patients has no detectable amount of 1 α,25(OH)$_2$D$_3$ (*5, 6*). We now show that nephrectomy does not abolish 1 α-hydroxylation in the pregnant rat, and these results provide indirect evidence for an extra-renal site of 1 α-hydroxylation during pregnancy.

Female Sprague-Dawley rats obtained at 2 to 3 months of age were fed a synthetic, vitamin D-free diet (*7*). Vitamin D deficiency was documented by the analysis of plasma for the concentration of 25OHD, using a modified radioreceptor assay (*8*). Plasma levels of 25OHD were below the low limit of detectability for the assay (0.5 ng/ml) after 6 weeks of this diet. After 8 weeks of the diet, the vitamin D-deficient rats were bred with normal males. The presence of spermatozoa in the vaginal aspirates was used to identify the first day of pregnancy. On day 21 of pregnancy, ^{3}H-(25,26)-25OHD$_3$ (5 I.U., 22 mCi/mg; Amersham/Searle) dissolved in 95 percent ethanol was administered by an intravenous injection to the pregnant rats. Some pregnant rats were nephrectomized under ether anesthesia immediately prior to the intravenous injection. Likewise, nonpregnant vitamin D-deficient rats, either nephrectomized or with intact kidneys, were injected intravenously with the same dose of ^{3}H-(25,26)-25OHD$_3$.

Six hours after the injection of ^{3}H-(25,26)-25OHD$_3$, rats were anesthetized with ether and bled by cardiac puncture. Maternal and fetal tissues were removed through an abdominal incision in the mother and microdissection of the fetuses. Plasma was separated from the red blood cells by centrifugation and was extracted with methanol:chloroform (2:1) for 1 hour (*9*). Other tissues were washed in tris buffer (*p*H 7.4, 4°C), homogenized, and extracted with methanol:chloroform. Chloroform fractions were dried under a stream of N_2. The lipid extracts were chromatographed on Sephadex LH-20 columns (2 by 30 cm) with a solvent system of chloroform:hexane (65:35) (*10*). An aliquot of each fraction was counted on a Beckman liquid scintillation counter using a counting solution of 0.5 percent 2,5-diphenyloxazole and 0.01 percent *p*-bis[2-(5-phenyloxazolyl)]benzene in toluene. Radioactivity recovered from these columns averaged 95 percent of the total extracted counts. Fractions migrating in the region of synthetic 1 α,25(OH)$_2$D$_3$ were rechromatographed on Sephadex LH-20 column (1 by 57 cm) with a solvent system of hexane:chloroform:methanol (90:10:10) (*11*). Fractions which again comigrated with synthetic 1 α,25(OH)$_2$D$_3$ were pooled, dried under N_2, and redissolved in hexane:isopropanol (87:13) (*12*). These pooled fractions were cochromatographed with synthetic metabolites (donated by M. Uskokovic, Hoffmann-La Roche, Nutley, New Jersey) on a high-pressure liquid chromatography system (HPLC) (Laboratory Data Control) using a microsilica column (4.6 mm by 25 cm; LDC Spherisorb) and a solvent system of hexane:isopropanol (87:13) at a flow of 1 ml/min and at 1700 pounds per square inch (*12*). Resolution of the vitamin D metabolites by this system is shown in Fig. 1. Radioactivity of each fraction was counted in the same manner described above. ^{3}H-1 α,25(OH)$_2$D$_3$ was prepared from ^{3}H-25OHD$_3$ in the chick homogenate system (*13*). The biosynthetic ^{3}H-1 α,25(OH)$_2$D$_3$ was purified by serial chromatography on Sephadex LH-20 columns and on microsilica in an HPLC system. Purity of the final product was established by comigration with synthetic 1 α,25(OH)$_2$D$_3$.

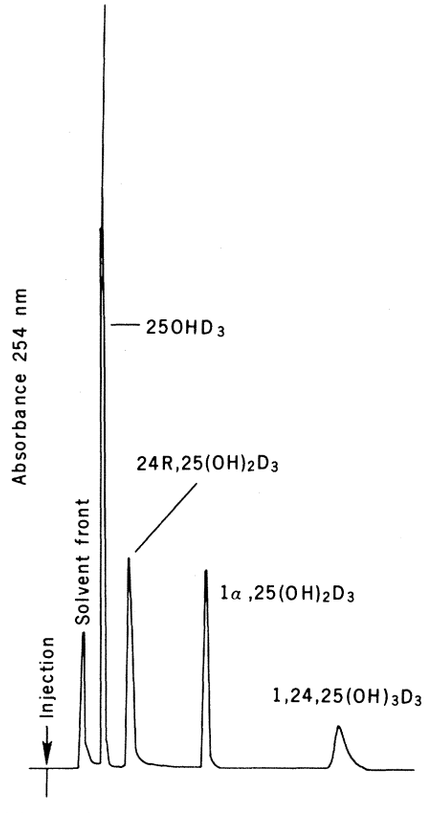

Fig. 1. Chromatographic separation of synthetic vitamin D_3 metabolites by HPLC. Nanogram amounts of each metabolite were dissolved in hexane:isopropanol (20 μl) and injected over a microsilica column (4.6 mm by 25 cm) (Spherisorb, Laboratory Data Control) using a mobile phase of hexane:isopropanol (87:13) at a flow of 1 ml/min at 1700 pounds per square inch, recording ultraviolet absorbance at 254 nm.

A radioactive peak eluting in the fractions numbered 42 to 58 was seen in the Sephadex LH-20 chromatograms of the plasma lipid extracts from the pregnant, vitamin D-deficient rats (Fig. 2A). Purified ^{3}H-1 α,25(OH)$_2$D$_3$ eluted from this column in the same fractions. Nephrectomy of the pregnant, vitamin D-

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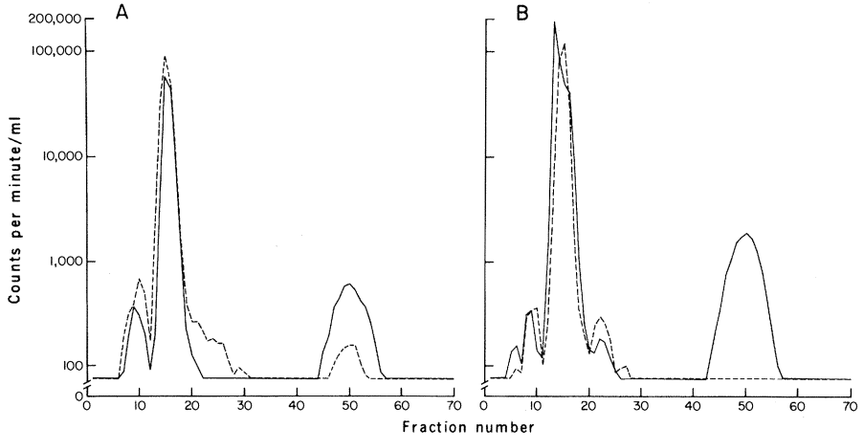

Fig. 2. Chromatogram of the plasma extracts from vitamin D-deficient rats, either pregnant or not, after intravenous injection of ^{3}H-25OHD$_3$. Plasma extracts were chromatographed over Sephadex LH-20 columns (2 by 30 cm, chloroform:hexane, 65:35), and samples of 5-ml fractions were collected for counting of the radioactivity. Chromatograms shown are representative examples of three separate experiments. (A) Elution patterns from pregnant rats with intact kidneys (—) or nephrectomized (- - - -); (B) elution patterns from nonpregnant rats with intact kidneys (—) or nephrectomized (- - - -).

deficient rats was associated with a reduction in the size of this radioactive peak, but the peak was not abolished (Fig. 2A). The chromatograms of the plasma lipid extracts from nonpregnant, vitamin D-deficient rats also contained a radioactive peak eluting in fractions numbered 42 to 58 (Fig. 2B). Nephrectomy of the nonpregnant, vitamin D-deficient rats was associated with the complete absence of a radioactive peak in fractions 42 to 58 (Fig. 2B). A comparison of the chromatograms revealed that the in vivo formation of 1 α,25(OH)$_2$D$_3$ was reduced but was not completely abolished by nephrectomy of the pregnant, vitamin D-deficient rats, in contrast to the results observed in the nonpregnant rats (Fig. 2, A and B).

Definitive identification of the radioactive peak still found in vivo after nephrectomy of pregnant, vitamin D-deficient rats was undertaken. Fractions 42 to 58 from the chromatograms of the pregnant, nephrectomized rats were pooled, dried under a stream of N$_2$, and applied to another Sephadex LH-20 column, 1 by 57 cm, in hexane:chloroform:methanol (90:10:10). The chromatogram from this second column showed a single, sharp peak which migrated in the same region as purified ^{3}H-1 α,25(OH)$_2$D$_3$. The fractions from this radioactive peak were pooled, dried under N$_2$, and redissolved in hexane:isopropanol (87:13). Injection of this material into the HPLC system revealed a single peak of radioactivity which comigrated with synthetic 1 α,25(OH)$_2$D$_3$ (Fig. 3).

Lipid extracts of the placental tissues, pooled fetal plasma, and the fetal kidneys removed from the nephrectomized mothers were also chromatographed as described above. The amount of ^{3}H-1 α,25(OH)$_2$D$_3$ formed in vivo was calculated from the recovered radioactivity of the extracts and the specific activity of the injected ^{3}H-25OHD$_3$. Based on these calculations maternal plasma after nephrectomy contained 36 pg/ml of 1α, 25(OH)$_2$D$_3$ while fetal plasma contained 8 pg/ml. Fetal kidneys contained an average level of 195 pg/g of 25OHD$_3$ but no detectable 1 α,25(OH)$_2$D$_3$. Placental tissue contained 28 pg/g of the dihydroxylated metabolite.

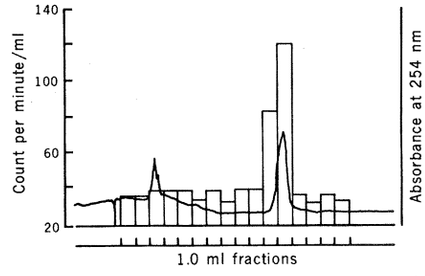

Fig. 3. Cochromatography of the plasma peak from nephrectomized, pregnant rats with synthetic 1 α,25(OH)$_2$D$_3$ on HPLC. The plasma peak represents the pooled fractions 42 to 58 from Sephadex LH-20 chromatography (2 by 30 cm column, chloroform:hexane, 65:35) of the plasma extracts from three nephrectomized, pregnant rats. These pooled eluates were rechromatographed over Sephadex LH-20 (1 by 57 cm column, hexane:chloroform:methanol), and the eluting fractions which comigrated with synthetic 1α, 25(OH)$_2$D$_3$ were pooled, dried under N$_2$, and redissolved in hexane:isopropanol. The plasma peak was injected over a microsilica column (4.6 mm by 25 cm; Spherisorb, Laboratory Data Control) with a mobile phase of hexane:isopropanol at a flow of 1 ml/min and at 1700 pounds per square inch. The ultraviolet absorbance (solid line) reveals three deflections: the point of injection, the solvent front, and synthetic 1 α,25(OH)$_2$D$_3$. The blocks represent counts per minute per milliliter of eluate from the HPLC system.

These studies confirm and extend existing concepts about the anatomic localization of the 1 α-hydroxylation of 25OHD$_3$. As expected, nephrectomy of vitamin D-deficient rats completely prevented the in vivo synthesis of 1α, 25(OH)$_2$D$_3$ (Fig. 2B). In contrast, nephrectomy of the pregnant, vitamin D-deficient rats reduced the formation of 1 α,25(OH)$_2$D$_3$ but it did not abolish this step completely as in the nonpregnant rats (Fig. 2, A and B). The radioactive metabolite which persisted in the circulation of the pregnant, nephrectomized rats was identified by cochromatography on HPLC with synthetic standards to be 1 α,25(OH)$_2$D$_3$ (Fig. 3). These results show that 1α-hydroxylation occurred at a site other than the maternal kidneys.

Lipid extracts of fetal kidneys pooled from three litters contained 25OHD$_3$ but no 1 α,25(OH)$_2$D$_3$. 1 α,25(OH)$_2$D$_3$ was still undetectable in the fetal kidney extracts after the administration of ^{3}H-25OHD$_3$ with a higher specific activity (44 mCi/mg) to the nephrectomized mothers. In contrast, when the maternal kidneys were intact and 1 α,25(OH)$_2$D$_3$ was present in maternal and fetal plasma in higher amounts, 1 α,25(OH)$_2$D$_3$ was observed in the fetal kidney extracts. The possibility that 1 α,25(OH)$_2$D$_3$ was present in minute amounts in the fetal kidneys after maternal nephrectomy remains as an unresolved issue. The present results are consistent with the concept of an extra-renal site of 1α-hydroxylation for 25OHD$_3$ in the pregnant rat. The anatomic origin of this synthetic site may be maternal, fetoplacental, or a combination of these.

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Oxytocin Receptors: Triggers for Parturition and Lactation?

Abstract. *Specific binding of tritiated oxytocin to uterine receptors of pregnant rats increases dramatically at term and is maximal during labor. In mammary glands the increase in binding is gradual, reaching a maximum during the lactation period. Concomitant changes in the sensitivity of the uterus and mammary gland to oxytocin indicate that the receptor concentration is of functional significance. Oxytocin receptors, therefore, may regulate the response of the target organs to circulating oxytocin and thereby control the onset of labor and lactation. Ovarian steroids participate in the regulation of oxytocin receptors in a manner as yet unclarified.*

The neurohypophysial hormone, oxytocin, causes contraction of uterine smooth muscle and has been used widely in obstetrics to induce labor. Because oxytocin-induced labor cannot be distinguished from spontaneous labor by the pattern of contractions, it was assumed for decades that labor is initiated by the release of oxytocin. Although oxytocin has been found in the maternal circulation throughout pregnancy (*1*), most observations suggest that oxytocin concentrations in the blood increase only during the final stages of labor, and not before (*2*). Therefore, parturition may not be triggered by an increase in oxytocin secretion, but by an increase in the sensitivity of the myometrium of the uterus to the hormone. Several studies, in fact, have shown that the reactivity of the myometrium to oxytocin is maximal at or near labor (*3*, *4*).

We have examined whether the increased sensitivity of the myometrium in pregnant rats is the result of an increase in the concentration of oxytocin receptors in the myometrium at the time of parturition. A sudden increase in the concentration of oxytocin receptors would support the concept that the receptors are the trigger for parturition.

Oxytocin also causes milk ejection in lactating animals by eliciting contractions of the myoepithelial cells surrounding the alveoli in mammary glands. In rats, oxytocin is essential for the removal of milk from the mammary glands (*5*), and the initiation of lactation also may depend on an increase in oxytocin receptors. Therefore, we have determined the concentration of oxytocin receptors in the mammary glands during pregnancy and lactation in the rat.

The specific binding of [^{3}H]oxytocin to particulate fractions from the rat mammary gland and myometrium was estimated as described (*6*). Scatchard analyses indicated that oxytocin was bound to myometrial particles with an apparent dissociation constant (K_d) of 1 to 2 n*M* throughout pregnancy. These values agree with those found in uterine particulate fractions from estrogen-treated rats (*6*). The metabolic breakdown of [^{3}H]oxytocin by uterine particles was generally uniform throughout pregnancy, never exceeding 30 percent after the 1-hour incubation period. The amount of oxytocin bound to myometrial particles was relatively high on day 1 of pregnancy (estrus), but decreased thereafter to near baseline levels by day 5 of pregnancy and remained low until the day of parturition, day 22 (Fig. 1A). Binding then increased rapidly and reached peak values during labor. Binding was re-

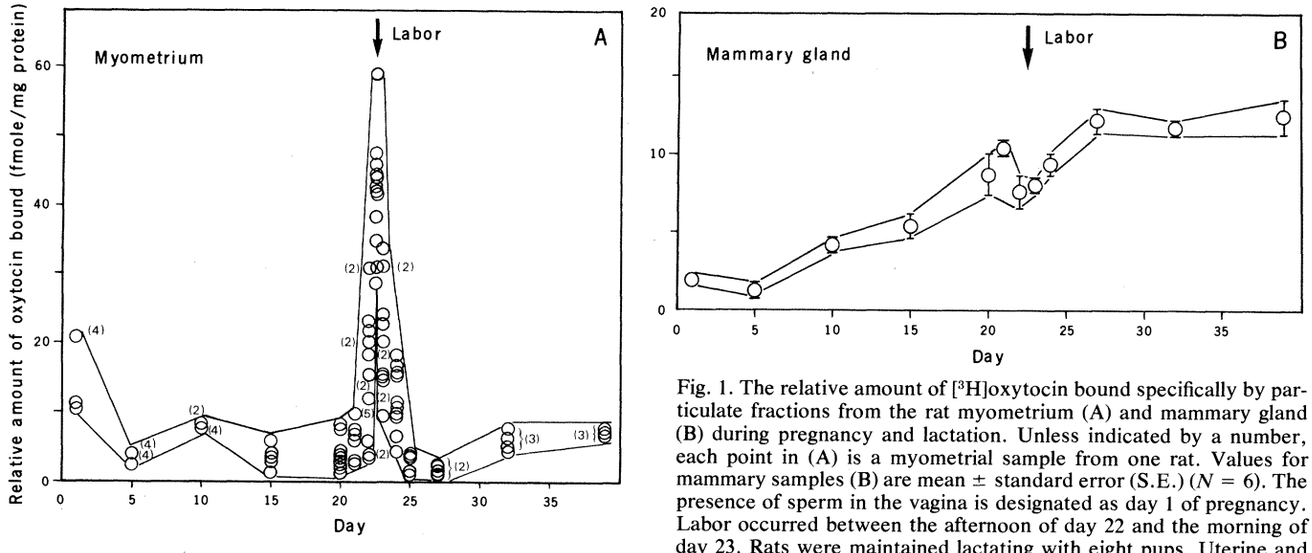

Fig. 1. The relative amount of [^{3}H]oxytocin bound specifically by particulate fractions from the rat myometrium (A) and mammary gland (B) during pregnancy and lactation. Unless indicated by a number, each point in (A) is a myometrial sample from one rat. Values for mammary samples (B) are mean ± standard error (S.E.) (N = 6). The presence of sperm in the vagina is designated as day 1 of pregnancy. Labor occurred between the afternoon of day 22 and the morning of day 23. Rats were maintained lactating with eight pups. Uterine and mammary samples from Sprague-Dawley rats (ARS, Madison) were taken between 1000 and 1200 hours. The myometrium was dissected free and homogenized in nine volumes of Tyrode solution as described (*6*). Particles sedimenting between 1,000*g* (10 minutes) and 48,000*g* (30 minutes) were assayed for binding activity. Each assay tube contained 1 mg of particulate protein, 0.5 n*M* [^{3}H]oxytocin (31 Ci/mmole, 452 I.U./mg, custom synthesized by Schwarz/Mann) in 250 μl of 50 m*M* tris-maleate buffer, *p*H 7.6, containing 5 m*M* $MnCl_2$ and 0.1 percent (weight to volume) gelatin. The amount of [^{3}H]oxytocin bound in the presence of 0.2 μ*M* nonradioactive oxytocin was considered to be nonspecific. Nonspecific binding never exceeded 25 percent of the amount of oxytocin bound specifically during labor. Specific binding was determined by subtracting the amount bound nonspecifically from the total radioactivity. Protein concentrations were determined by the method of Lowry *et al.* (*20*) with bovine serum albumin as the standard.

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