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25-HYDROXYVITAMIN D AND 24,25-DIHYDROXYVITAMIN D IN MATERNAL PLASMA, FETAL PLASMA AND AMNIOTIC FLUID IN THE RAT

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SUMMARY

At the end of gestation plasma levels of 25-hydroxyvitamin D and 24,25-dihydroxyvitamin D were lower in pregnant than non pregnant female rats. In fetal plasma, concentrations of both metabolites were higher than in maternal plasma. This materno-fetal gradient led us to compare maternal and fetal plasma binding abilities. Fetal plasma was half as potent in binding 25-hydroxyvitamin D as maternal plasma. In fetal plasma binding was mainly due to the plasma vitamin D binding protein. On the other hand this study clearly showed that amniotic fluid contained 25-hydroxyvitamin D and 24,25-dihydroxyvitamin D. In addition this fluid was found to possess vitamin D-metabolite binding activity. The molecule responsible for this has been identified as the plasma vitamin D binding protein.

INTRODUCTION

Vitamin D undergoes a series of functional activations in order to fill its biological role (1). 25-hydroxyvitamin D (25-OHD), the major circulating metabolite, is considered as a prehormone susceptible to further hydroxylation in positions 1 or 24, thus forming 24,25-dihydroxy-vitamin D (24,25-$(OH)_2$D). The exact role of the latter, which is the

Abbreviations : 25-OHD : 25-hydroxyvitamin D ; 25-OH$[^3H]D_3$: tritiated 25-OHD_3 ; 24,25-$(OH)_2$D : 24,25-dihydroxyvitamin D ; 24,25-$(OH)_2[^3H]D_3$: tritiated 24,25-$(OH)_2D_3$; H.P.L.C. : high performance liquid chromatography ; DBP : serum vitamin D binding protein.

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predominant dihydroxylated metabolite circulating in blood, has not yet been well established. Both 25-OHD and 24,25-$(OH)_2D$ are known to be transported in rat plasma partially bound to the specific vitamin D binding protein (DBP) (2,3,4).

There is now evidence that vitamin D metabolism alters during pregnancy (5-10). Clear differences also exist between mother and fetus. However, most of the data have been obtained by indirect approaches such as injecting labelled metabolites into vitamin D-deprived or nephrectomized pregnant rats (7-10). The present study was undertaken to establish, by the direct means of a competitive protein-binding assay, the plasma concentrations of the above two predominant circulating vitamin D metabolites in both maternal and fetal blood from normal pregnant rats. In addition, maternal and fetal plasma were compared for their vitamin D binding properties.

Vitamin D metabolite measurements and binding experiments were extended to amniotic fluid, since it contains several steroids and hormones involved in fetal development (11).

MATERIAL AND METHODS

Animals. Six 10-week-old pregnant Wistar rats from Lessieux (France) arrived in the laboratory at day 15.5 of gestation and were fed ad libitum with a normal diet containing 4000 IU vitamin D_3 per kilo. Investigations were made on day 19.5 of gestation. Under light ether anaesthesia of the mother, a cesarian section was performed. Amniotic fluids were carefully collected by aspiration and all samples with possible blood contamination discarded. Blood samples were obtained from fetus axillary vessels and maternal aorta. In each litter amniotic fluids and fetal blood were pooled. Five non-pregnant 11-week-old females were bled as controls. All samples were centrifuged for 10 min. at 2500 g and 4°C ; plasma and amniotic fluids were placed in glass tubes and kept frozen (-20°C).

Materials. 25-hydroxy [26(27)-methyl-^{3}H] cholecalciferol : (25-OH[^{3}H]D_3 -, 9 Ci/mmol) and 24,25-dihydroxy [23,24(n)-^{3}H] cholecalciferol : 24,25-$(OH)_2$[^{3}H]D_3, 82 Ci/mmol) were purchased from Amersham (G.B.). They were purified before use by high performance liquid chromatography (HPLC). The specific activity of 24,25-$(OH)_2$[^{3}H]D_3 was lowered to 9 Ci/mmol with unlabelled 24,25-$(OH)_2D_3$. Unlabelled 25-OHD_3 was a gift from Dr Six (Laboratoires Roussel, France), and 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ were kindly supplied by Dr Uskokovic (Hoffman-La Roche, U.S.A.). The concentrations of unlabelled steroids were confirmed spectrophotometrically (ε=18700).

High performance liquid chromatography was performed through a μC18 Bondapak (Waters) reverse plasma column equipped with a Waters 6000 A

solvent pump and a Waters U6K injector. The elution solvent, methanol/water 8/2 was run at a rate of 2 ml/min. Calibration was done with unlabelled steroids monitored by their absorption at 254 nm.

Radioactivity was measured in an Intertechnique SL 40 liquid scintillation counter using Lipoluma (Lumac) with 45% efficiency for organic samples, and Picofluor (Packard) for aqueous solutions (35% efficiency).

25-OHD and 24,25(OH)$_2$D assays. For vitamin D metabolite separation, a new method was devised consisting of direct purification of a single sample extract by HPLC. To estimate recovery through the extraction and chromatography, 25-OH[^{3}H]D$_3$ and 24,25-(OH)$_2$[^{3}H]D$_3$ (12000 dpm) were added to the sample (1 or 0.5 ml) before extraction. Metabolites were extracted by precipitating proteins in ethanol up to a final concentration of 90%. The alcoholic extract was evaporated to dryness under vacuum, taken up in peroxide-free di-ethyl ether and washed with 0.1 M Na_2HPO_4, pH 10. This treatment allowed direct introduction of the organic phase into the HPLC column. The well separated 24,25-(OH)$_2$D and 25-OHD fractions (respective retention times : 5 and 12 min.) were collected with respective recoveries (mean ± SEM) of 52±1.5% (n = 37) and 59±1.5% (n = 37). For each sample, a duplicate determination was made by radiocompetitive assay using rachitic rat serum (12) in barbital acetic buffer, pH 8.6, containing human serum albumin and lipoprotein. Bound and free steroids were separated by dextran charcoal suspension. All values were corrected for non-specific binding. In our standard curves, the range for correct values is from 0.1 ng to 1 ng sterol/tube. Care was taken to assay the three samples from each mother in the same batch.

This modified assay permits better control of the purification step compared to open silicagel columns. However the possibility of partial cross-contamination of the 24,25-(OH)$_2$D fractions by interfering substances like the recently described 25-OHD-26-23 lactone (13) cannot be ruled out.

Results were analyzed statistically using the Student's "t" test.

Binding characterization. Plasma and amniotic fluid (1 to 3 mg protein/ml) were incubated with 25-OH[^{3}H]D (100.000 dpm) for 16 hours at 4°C and 25-OH[^{3}H]D$_3$ binding was then analyzed.

Polyacrylamide gel electrophoresis was run at 4°C (3.5 h ; 17 V/cm) using 7 or 10% polyacrylamide in Tris-glycine buffer, pH 8.6. After migration, cylindrical gels were cut longitudinally. One half was stained with Coomassie Blue and the other half, sliced into 2 mm fractions extracted with 0.5 ml ethanol and counted for radioactivity.

Gel chromatography was performed in a column (1.5 x 25 cm) of G 75 Sephadex gel, eluted with buffer (50 mM K_2HPO_4, 50 mM KCl, pH 7.4) at 4°C, at a rate of 0.5 ml/mm. 0.5 ml fractions were collected and counted for radioactivity.

The sucrose density gradient (10% to 30% w/w) was centrifuged at 4°C in a Beckmann SW60 Ti rotor (16 h ; 485 000 g). Fractions were collected from the top and counted for radioactivity. ^{14}C-ovalbumin (3,6S) and ^{14}C-bovine serum albumin (4,6S) obtained from NEN were used as calibration standards.

Dialysis experiments. We used the batchwise equilibrium dialysis technique of Pearlman and Crepy using a suspension of Sephadex G25 gel as the semi-permeable membrane (14). Experiments were performed in a phosphate buffer, pH 7.4, with 0.1% human serum albumin and 10% ethanol in order to reduce non-specific absorption of 25-OH[^{3}H]D$_3$ (100.000 dpm). 1/P binding indexes (L/g) were calculated, with P as the protein concentration corresponding to an equilibrium ratio of 25-OHD$_3$ unbound/25-OHD$_3$ bound = 1.

Protein contents were determined by a modified version of Lowry's method (15).

RESULTS AND DISCUSSION

Maternal and fetal plasma. Levels of 25-OHD and 24,25-$(OH)_2D_3$ in the maternal plasma of pregnant rats on day 19.5 of gestation were lower than those in plasma of control non-pregnant females, as follows : 25-OHD : 7.4±0.5 vs 14.0±0.8 ng/ml ($p<0.001$) ; 24,25-$(OH)_2D$: 7.0±1.1 vs 12.6±2.1 ng/ml ($p<0.05$) (figure 1). The physiological occurence of such decreases at the end of gestation completely agrees with other recent findings (5).

Fetal plasma values for 25-OHD and 24,25-$(OH)_2D$ were higher than maternal values, respectively : 17.3±1.4 vs 7.4±0.5 ng/ml ($p<0.001$) for 25-OHD and 14.0±2.0 vs 7.0±1.1 ng/ml ($p<0.02$) for 24,25-$(OH)_2D$ (figure 1). At such concentrations, 25-OHD probably serves as a substrate for the fetal 1α-hydroxylase, since rat and rabbit fetal kidney homogenates have been shown to produce 1,25-$(OH)_2D_3$ (8,16). On the other hand, the high levels of 24,25-$(OH)_2D$ found here in fetal plasma may be related to the ability of rat fetus to accumulate this metabolite (7,8,10). This seems to indicate that 24,25-$(OH)_2D$ plays a part in fetal development, possibly in bone metabolism (17-20).

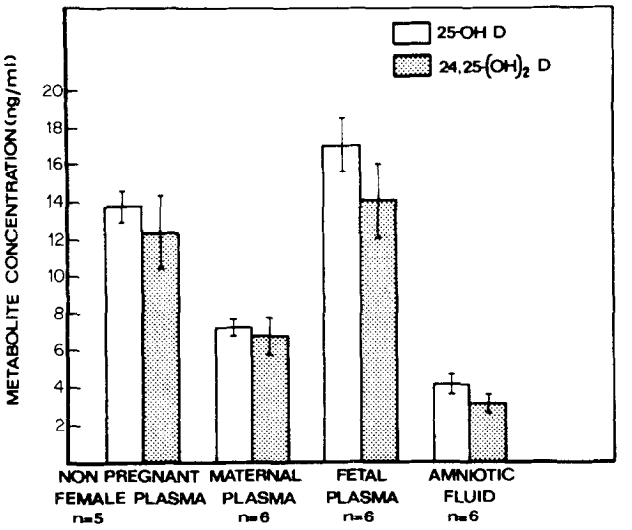

Figure 1 : Concentrations of 25-hydroxyvitamin D and 24,25-dihydroxyvitamin D in maternal plasma, fetal plasma and amniotic fluid from pregnant rats on day 19.5 of gestation. Values are given as means ± S.E.M.

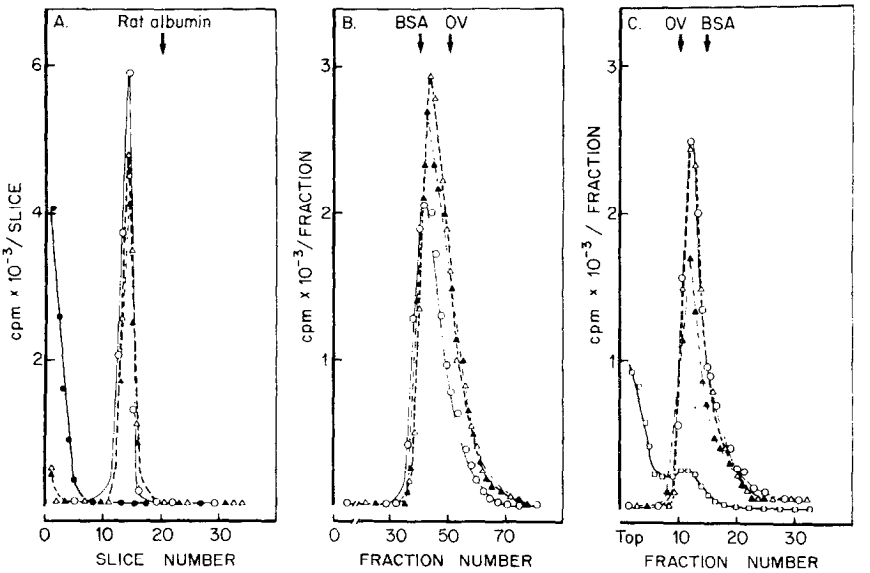

Figure 2 : Biochemical characterization of 25-OHD binding : typical profiles of maternal plasma (Δ), fetal plasma (▲) and amniotic fluid (o) previously incubated with 25-OH[^{3}H]D_3 alone or in presence of a large excess of unlabelled 25-OHD (□) ; (●) 25-OH[^{3}H]D_3 alone.
A : Electrophoresis on 10% polyacrylamide gel ;
B : Gel filtration on G75 Sephadex gel column ;
C : Sucrose (10-30% w/w) density gradient centrifugation.

The materno-fetal gradient of both metabolites might be due to binding properties peculiar to fetal plasma, whether or not 25-OHD and/or 24,25-$(OH)_2D$ are produced in the fetus. This prompted us to compare vitamin D metabolite-binding by fetal and maternal plasma respectively (figures 2 and 3).

Qualitative studies are illustrated in figure 2. Three different biochemical methods showed that the 25-OH[^{3}H]D_3 bound to fetal or maternal plasma exhibits a single peak, suggesting that only one protein is mainly responsible for the binding. This peak is similar in both maternal and fetal plasma. It has an electrophoretic mobility in the α_2-globulin region (pannel A). Gel filtration (pannel B) gives an elution between that of BSA and ovalbumin, indicating an approximate molecular weight of 50.000. The sedimentation coefficient (pannel C) in the sucrose density gradient is 4 S. These physico-chemical parameters are similar to those obtained with adult male rat plasma and are consistent with those of rat DBP (4).

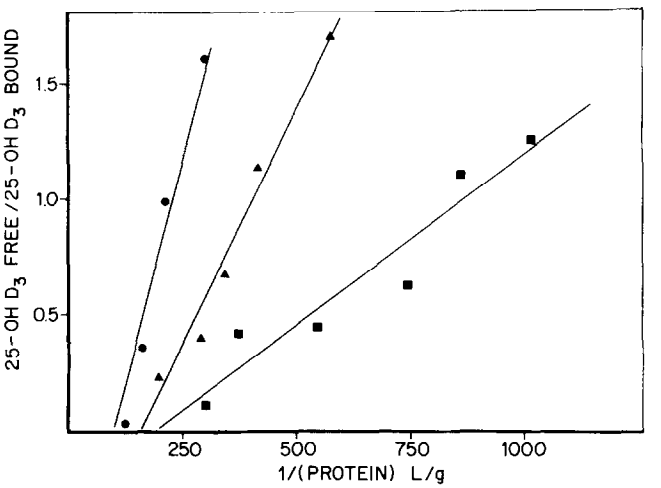

Figure 3 : Dialysis experiments : determination of 1/P binding indexes of 25-OH[^{3}H]D_3 with maternal plasma (■), fetal plasma (▲) and amniotic fluid (●).

Dialysis experiments (figure 3) indicate that the 1/P binding indexes are 400 L/g for fetal plasma and 870 L/g for maternal plasma. Thus, fetal plasma is half as potent in binding 25-OHD as maternal plasma. This lower fetal plasma binding ability may be related to the immunoreactive DBP level, which is lower in fetal than maternal plasma (22). Since rat α_1-feto protein is the main protein component of fetal plasma and possesses oestrogen steroid binding properties (23,24), we tested its ability to bind vitamin D metabolites. For both 25-OHD and 24,25-$(OH)_2$D, pure rat α_1-feto protein (25) exhibited a 1/P index of 10 L/g. Although low, this value is not negligible but contrasts with the 1/P of 400 L/g for fetal plasma.

Thus, in fetal plasma, DBP plays the main role in vitamin D metabolite binding and the participation of other fetal plasma proteins seems of less importance. In addition, fetal binding properties alone therefore do not explain the metabolite gradient.

Amniotic fluid. For the first time, the existence of 25-OHD and 24,25-$(OH)_2$D is clearly shown in amniotic fluid (figure 1). Since the protein contents of amniotic fluid samples were estimated to be low (1.21±0.04 mg

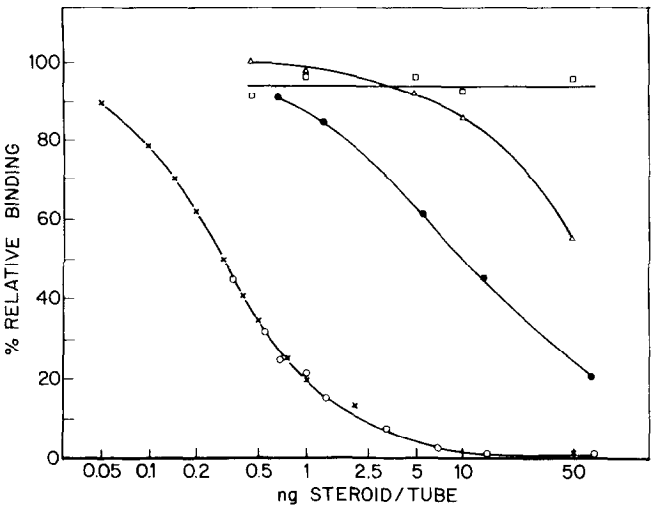

Figure 4 : Displacement curves obtained with amniotic fluid : amniotic fluid was incubated with 25-OH[^{3}H]D_3 in the presence of variable amounts of different steroids ; x—x 25-OHD_3, o—o 24,25-$(OH)_2D_3$, ●—● 1,25-$(OH)_2D_3$, Δ—Δ vitamin D_3, □—□ corticosterone.

prot./ml, n = 6), blood contamination is unlikely to account for the values observed. Levels of 25-OHD (4.3±0.5 ng/ml) and 24,25-$(OH)_2$D (3.4±0.5 ng/ml) are elevated in relation to the poor lipid content of amniotic fluid. Nevertheless, they are lower than those found in maternal and fetal plasma. Vitamin D metabolites in amniotic fluid may be of importance in normal fetal development, either as a source for fetal needs, or as a fetal excretion pathway.

By analogy with plasma, we tested amniotic fluid for its vitamin D metabolite binding. Amniotic fluid bound 25-OHD with a 1/P index of 220 L/g (figure 3). The activities of the main vitamin D_3 metabolites in displacing 25-OH[^{3}H]D_3 from amniotic fluid were defined. Figure 4 shows the competition displacement curves : at 50% inhibition, potencies are 100% for 25-OHD_3 and 24,25-$(OH)_2D_3$, 3% for 1,25-$(OH)_2D_3$ and less than 0.5% for vitamin D_3 ; corticosterone does not compete. Similar relative potencies are known for rat plasma DBP (2,3,4). Furthermore, the present qualitative studies (figure 2) confirmed that DBP is the macromolecule responsible for binding in amniotic fluid. The evidence that this fluid contains several

steroids together with their specific binding proteins can therefore now be extended to vitamin D and DBP.

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