

Lower affinity for substrate for extrarenal synthesis of calcitriol in chronic uremia

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Lower affinity for substrate for extrarenal synthesis of calcitriol in chronic uremia. Previous studies from our laboratory have shown that anephric patients have very low, but detectable, levels of $1,25(\text{OH})_2\text{D}_3$ (calcitriol) that can be increased to normal by administration of large doses of $25(\text{OH})\text{D}_3$. The report of 1α -hydroxylase activity in pig liver with an affinity for substrate significantly lower than that of the renal enzyme, led us to use the rat as an experimental model to further clarify the need of supraphysiological levels of $25(\text{OH})\text{D}_3$ to correct calcitriol deficiency in chronic uremia. We have measured $1,25(\text{OH})_2\text{D}_3$ production by rat liver. Cytosol free liver homogenates (CFH) from normal rats were incubated with $25(\text{OH})\text{D}_3$ and the production of $1,25(\text{OH})_2\text{D}_3$ was measured using the thymus radioreceptor assay after solid phase C18 extraction and HPLC purification of the samples. $1,25(\text{OH})_2\text{D}_3$ production was linear up to 30 minutes and a CFH protein concentration up to 20 mg. Saturability was attained for a substrate concentration of approximately $60 \mu\text{M}$. Ketoconazole, a cytochrome P450 inhibitor, blocked calcitriol production in a dose dependent fashion. Total inhibition of the liver 1α -hydroxylase was achieved with $180 \mu\text{M}$ ketoconazole. We next compared the kinetics of the 1α -hydroxylases of normal and uremic rat livers. Maximal velocities were not statistically different ($139.6 \pm 22.3 \text{ pg/mg/min}$ for normals and $217.1 \pm 73.3 \text{ pg/mg/min}$ for uremic rats). However, the apparent K_m was $35.9 \pm 3.2 \mu\text{M}$ for uremic animals, significantly higher ($P \leq 0.001$) than that of normal rats ($16.6 \pm 0.7 \mu\text{M}$). These results demonstrate that: (1) 1α -hydroxylase is expressed in rat livers; (2) the liver enzyme appears to be a cytochrome P450 mixed function oxidase; (3) the affinity of the liver enzyme for $25(\text{OH})\text{D}_3$ is significantly reduced in uremia, a finding which may explain the need of supraphysiological levels of substrate to normalize serum calcitriol in anephric humans.

In the past decade, there has been increasing evidence that the kidney is not unique in metabolizing $25(\text{OH})\text{D}_3$ to $1,25(\text{OH})_2\text{D}_3$. Previous studies in our laboratory have shown that anephric dialysis patients have low basal serum concentrations of calcitriol ($5.5 \pm 1.2 \text{ pg/ml}$) which suggest the existence of extrarenal production of calcitriol [1]. In these patients, a significant correlation between the circulating levels of $25(\text{OH})\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$ was found. Their serum $1,25(\text{OH})_2\text{D}_3$ increased to normal levels only when serum $25(\text{OH})\text{D}_3$ approached supraphysiological concentrations. This suggested a lower affinity or a decreased availability of substrate to the extrarenal 1α -hydroxylase(s).

In the last ten years numerous reports have shown the

capacity of different cells/tissues to synthesize calcitriol *in vitro* [2–11]. Recently, Hollis demonstrated 1α -hydroxylase activity in porcine hepatic tissue [12]. The K_m of the liver enzyme ($17 \mu\text{M}$) is significantly higher than that of the pig (445 nM) [13] and rat renal (890 nM) 1α -hydroxylases [14]. The lower affinity for $25(\text{OH})\text{D}_3$ of the hepatic 1α -hydroxylase could account for the need of supraphysiological $25(\text{OH})\text{D}_3$ levels to normalize serum calcitriol in anephric individuals.

To further investigate this possibility, we have examined the rat as an experimental model for hepatic $1,25(\text{OH})_2\text{D}_3$ production. We have confirmed that 1α -hydroxylase is expressed in the liver of normal rats, and we have characterized the kinetic of this enzyme. We compared the properties of the hepatic 1α -hydroxylase in normal rats to that in rats with chronic renal failure.

Methods

$25(\text{OH})\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$ were provided by Dr. Milan Uskokovic (Hoffman-LaRoche, Nutley, New Jersey, USA). $1,25(\text{OH})_2[26,27\text{-methyl-}^3\text{H}]\text{cholecalciferol}$ (SA: 160 to 180 Ci/mmol) and $25\text{-hydroxy-}[26,27\text{-methyl-}^3\text{H}]\text{cholecalciferol}$ (SA: 10 to 20 Ci/mmol) were obtained from Amersham (Arlington Heights, Illinois, USA). Dithiothreitol, N,N'-diphenylethylenediamine, nicotinamide adenine dinucleotide phosphate and isocitrate were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Coomassie blue protein assay reagent was obtained from Bio-Rad (Richmond, California, USA); C18 cartridges and all HPLC solvents from Fischer Scientific (Fair Lawn, New Jersey, USA).

Three-month old female Sprague-Dawley rats, 200 to 320 grams body weight, were fed a normal diet (#5001, Ralston-Purina, St. Louis, Missouri, USA), containing 1.0% calcium and 0.4% phosphorus. Uremia was induced by 5/6 nephrectomy. Seven to eight weeks after surgery the rats were sacrificed. The livers were removed for further preparation and the serum was retained for blood chemistry determinations.

The livers were perfused with ice-cold isotonic NaCl, minced and homogenized in four volumes of ice-cold buffer (50 mM; Na_2HPO_4 [pH = 7.4]; 0.25 M sucrose and 0.5 mM EDTA) using a polytron PT20 disrupter (Kinematica, Switzerland). Five milliliter aliquots of homogenate were stored at -70°C until assayed. Immediately before assaying, the liver homogenates were thawed and centrifuged at $100,000 \times g$ for one hour, as described by Hollis [12]. Cytosol-free homogenates were prepared by resuspending the resulting pellets to the original

volume using a buffer containing 50 mM Na₂HPO₄ (pH = 7.4); 125 mM sucrose; 5 mM KCl; 2 mM MgCl₂; 2 mM isocitrate; 1 mM NADPH; 3 mM EDTA; 1 mM DTT; 10 μM N,N' diphenylethylenediamine (DPED) to prevent free radical generation. The protein concentration of the CFH was measured by the method of Bradford [15] with ovalbumin as the standard.

The CFH was incubated in twelve-well plates at 37°C in an atmosphere containing 95% air, 5% CO₂. The final assay incubation volume was 2 ml. The reaction was initiated by adding 25(OH)D₃ in 20 μl ethanol to the wells and was terminated by adding 2 ml acetonitrile. Approximately 1000 cpm of [³H]-1,25(OH)₂D₃ were added to each well to estimate recoveries. The vitamin D metabolite fraction was obtained by C18 cartridge extraction using the procedure described by Reinhardt et al [16]. The fraction eluting with acetonitrile was dried under nitrogen and subjected to normal phase HPLC, using a Zorbax-Sil column (Phenomenex, St. Torrance, California, USA) and methylene chloride:isopropanol (96:4) at a flow rate of 2 ml/min. The 1,25(OH)₂D₃ fraction was collected, dried under nitrogen and resuspended in 200 μl ethanol. Average recoveries were 60 to 80%. 1,25(OH)₂D₃ production was quantitated using the calf thymus radioreceptor assay [16]. In order to confirm the identity of the putative 1,25(OH)₂D₃, the 1,25(OH)₂D₃ fraction from four samples, obtained from the HPLC system described above, was subjected to two additional HPLC systems: (1) normal phase HPLC using hexane:isopropanol (93:7) at 2 ml/min flow rate, and (2) reverse phase HPLC using a Zorbax-ODS column (DuPont Inc, Wilmington, Delaware, USA), with methanol:water (80:20) at 2 ml/min as solvent. The 1,25(OH)₂D₃ fraction was again dried under nitrogen, resuspended in 200 μl ethanol and quantitated using the radioreceptor assay of Reinhardt et al [16].

Zero time controls for endogenous 1,25(OH)₂D₃ present in the CFH were performed by adding acetonitrile to the reaction buffer before the CFH and the substrate. The 1,25(OH)₂D₃ levels measured in these controls were used to correct 1,25(OH)₂D₃ production of all 1,25(OH)₂D₃ determinations. Negative controls for non-enzymatic oxidation of 25(OH)D₃ consisting of both CFH boiled for three minutes and plates without protein were submitted to the same 25(OH)D₃ incubation reaction. 1,25(OH)₂D₃ levels in these negative controls did not differ from the zero time controls described above, and represented 0 to 3% of the maximal 1,25(OH)₂D₃ production (average V_{max}).

To determine K_m and V_{max}, liver CFH were incubated (in duplicate) with five different substrate concentrations from 0 to 180 μM 25(OH)D₃ and the rate of 1,25(OH)₂D₃ production was measured. K_m and maximal velocity were obtained from a single plot of the data.

The amount of DBP in the CFH of normal and uremic animals was estimated by quantitation of maximal specific 25(OH)D₃ binding. We measured competitive displacement of [³H]-25(OH)D₃ from CFH by radioinert 25(OH)D₃ using equilibrium ligand-binding conditions [17]. Two hundred and forty fmoles of [³H]-25(OH)D₃ dissolved in 10 μl ethanol was mixed either with 10 μl ethanol or with 200 molar excess (48,000 fmol) radioinert 25(OH)D₃ in 10 μl ethanol. Five hundred microliters of CFH diluted 1:10 or 1:20 [a concentration range which includes those used to determine 1,25(OH)₂D₃ production] with freshly prepared barbital-acetate buffer (0.05 M Na barbital-acetate, pH =

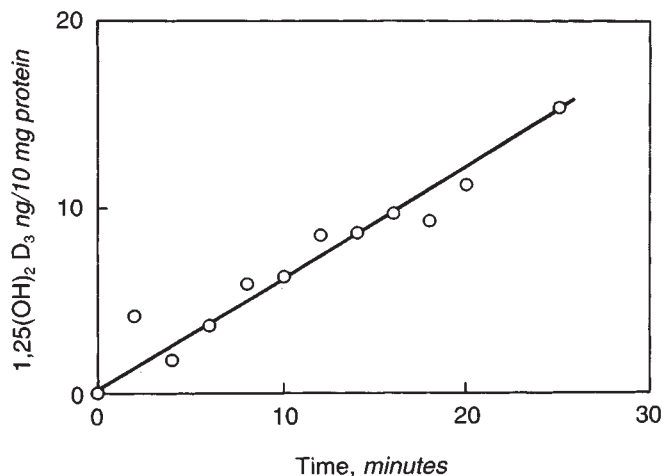


Fig. 1. Time course for 1,25(OH)₂D₃ production by rat liver CFH at a substrate concentration of 60 μM. Each point represents the mean of duplicate determinations.

8.6, 12 mM dithiothreitol, 0.1% BSA) were added and the samples were incubated at 0°C for two hours. Two hundred microliters of dextran-charcoal (2% Norit Charcoal Decolorizing Neutral, 2% T70 dextran in barbital-acetate buffer freshly prepared and stirred well before addition) was used to separate bound and free [³H]-25(OH)D₃. The assay tubes were vortexed and centrifuged at 1500 × g for 15 minutes at 0 to 5°C. Five hundred microliters of supernatant were collected and counted for tritium. The specific binding of 25(OH)D₃ to DBP [and possibly other 25(OH)D₃ binding proteins present in the CFH] was calculated using the formula O – E/T – E, where E is the cpm in the samples containing an excess of nonradioactive 25(OH)D₃, T is total counts, and O is the cpm in the samples containing no radioinert 25(OH)D₃.

Creatinine was determined using a Multistat 111 Plus (Instrumentation Laboratories, Boston, Massachusetts, USA). Calcium was determined by atomic absorption spectrophotometry (Perkin-Elmer, Model 503, Norwalk, Connecticut, USA). Serum 1,25(OH)₂D₃ was measured following the extraction procedure developed by Hollis [18] and the receptor assay of Reinhardt et al [16].

Student's *t*-test was used to quantitate statistical differences between experimental groups. Values were expressed as the mean ± SEM.

Results

The time course for conversion of 25(OH)D₃ to 1,25(OH)₂D₃ is shown in Figure 1. The reaction was linear up to 30 minutes. A 10 minute incubation was used in all further assays unless otherwise specified. Protein dependence is depicted in Figure 2. 1,25(OH)₂D₃ production was linear when amounts up to 20 mg protein of the CFH were used; 7.5 mg of protein were used in further experiments.

1,25(OH)₂D₃ production versus substrate concentration is shown in Figure 3. Saturation was reached at approximately 60 μM 25(OH)D₃.

Quantitation of 1,25(OH)₂D₃ production resulted in an average of 7.1 ng 1,25(OH)₂D₃ (average recovery: 69.2%) for the samples subjected to one HPLC purification and a mean of 6.9

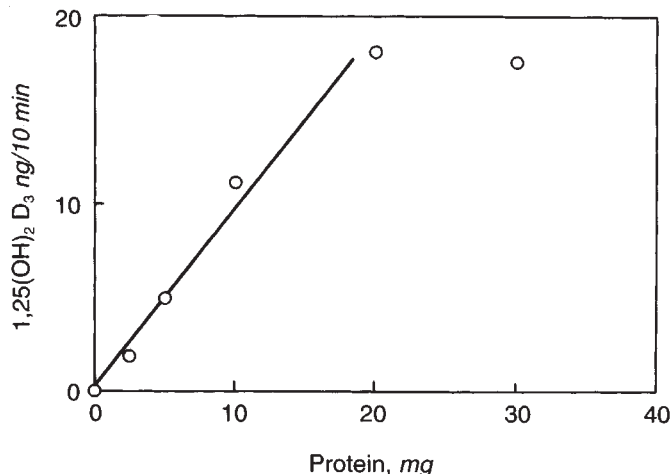


Fig. 2. Effect of protein concentration on $1,25(\text{OH})_2\text{D}_3$ production by rat liver CFH. Substrate concentration was $60 \mu\text{M}$. Each point represents the mean of duplicate determinations.

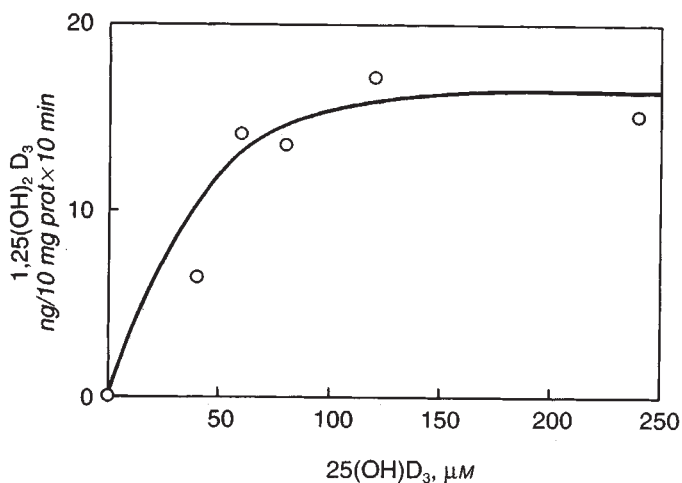


Fig. 3. Effect of substrate concentration on $1,25(\text{OH})_2\text{D}_3$ production by rat liver CFH. Each point represents the mean of duplicate determinations.

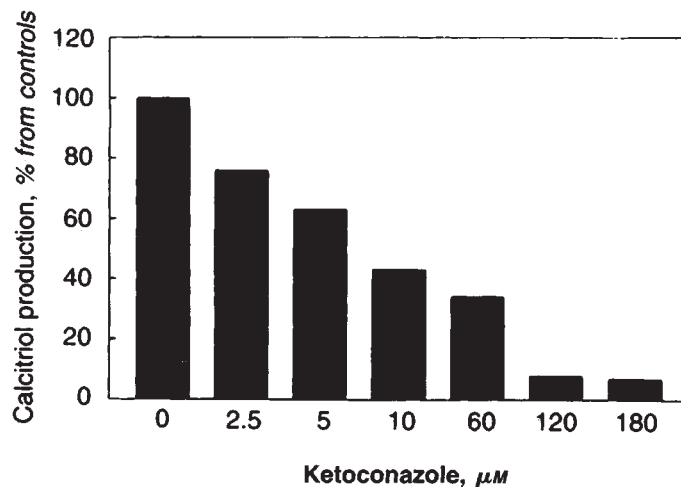


Fig. 4. The effect of ketoconazole concentration on the production of $1,25(\text{OH})_2\text{D}_3$ by rat liver CFH. Bars represent the mean of duplicate determinations.

Table 1. Body weight and serum concentrations of calcium, phosphorus, BUN, creatinine and $1,25(\text{OH})_2\text{D}_3$ in normal and uremic rats

	Normal (N = 7)	Uremic (N = 7)
Body weight g	262 ± 11.2	228.1 ± 10.3^a
Ca mg/dl	10.1 ± 0.1	10.3 ± 0.2
P mg/dl	4.6 ± 0.5	5.5 ± 1.5
BUN mg/dl	14.9 ± 1.3	46.5 ± 7.5^b
Creatinine mg/dl	0.6 ± 0.03	1.3 ± 0.1^b
$1,25(\text{OH})_2\text{D}_3$ pg/ml	33.2 ± 2.4	14.8 ± 2.9^b

Values are expressed as mean \pm SEM.

^a $P < 0.05$

^b $P < 0.01$

ng of $1,25(\text{OH})_2\text{D}_3$ (average recovery: 60.7%) for those undergoing three HPLC purification steps. This suggests that the compound eluting with [^3H]- $1,25(\text{OH})_2\text{D}_3$ using the methylene chloride:isopropanol system is authentic $1,25(\text{OH})_2\text{D}_3$. In all subsequent experiments, determination of $1,25(\text{OH})_2\text{D}_3$ production was performed after only one HPLC purification using methylene chloride:isopropanol.

Figure 4 shows the dose dependent inhibition of $1,25(\text{OH})_2\text{D}_3$ production by ketoconazole. Ketoconazole, at a concentration of $2.5 \mu\text{M}$ inhibited $1,25(\text{OH})_2\text{D}_3$ production by 23.6%. Total inhibition was achieved at a concentration of $180 \mu\text{M}$.

K_m and V_{max} of rat liver 1α -hydroxylase were measured in seven normal and seven uremic rats. The mean \pm SEM of body weight, and serum levels of calcium (Ca), phosphorus (P), blood urea nitrogen (BUN) and creatinine are shown in Table 1. There were no significant differences in the serum calcium and phosphorus between the two groups. As expected, in uremic ani-

mals, BUN and creatinine were significantly higher and serum $1,25(\text{OH})_2\text{D}_3$ significantly lower than in normal rats. Individual values for K_m and V_{max} are shown in Table 2. In normal rats, average K_m and V_{max} were $16.6 \pm 0.7 \mu\text{M}$ and 139.6 ± 22.3 pg of $1,25(\text{OH})_2\text{D}_3/\text{mg protein}/\text{min}$, respectively. In chronic uremia, the apparent K_m was $35.9 \pm 3.2 \mu\text{M}$, significantly higher ($P \leq 0.001$) than that of normal animals. The average value for V_{max} in uremic rats was 217.0 ± 73.3 pg $1,25(\text{OH})_2\text{D}_3/\text{mg protein}/\text{min}$. Due to the variation in V_{max} determinations in chronic renal failure, there was no significant difference in this parameter between normal and uremic rats.

There was no significant difference in the maximal specific binding of $25(\text{OH})\text{D}_3$ to DBP and other $25(\text{OH})\text{D}_3$ binding proteins in the CFH from normal and uremic animals (normal: 144.0 ± 6.0 fmol $25(\text{OH})\text{D}_3/\text{mg prot.}$ ($N = 6$) vs. uremic: 141.0 ± 6.5 fmol $25(\text{OH})\text{D}_3/\text{mg protein}$, $N = 5$).

Discussion

Extrarenal generation of calcitriol in humans has been conclusively demonstrated in sarcoidosis [19], pregnancy [20] and chronic renal failure [1]. In chronic uremia, the contribution of extrarenal sources of calcitriol to serum $1,25(\text{OH})_2\text{D}_3$ concentration is insignificant as proven by the low circulating levels of

Table 2. Individual values for K_m (μM) and V_{max} [pg of $1,25(\text{OH})_2\text{D}_3/\text{mg prot}/\text{min}$] determinations in liver homogenates from 7 normal and 7 uremic rats

K_m		V_{max}	
Normal	Uremic	Normal	Uremic
19	21	144	65.3
17	29	104	112
16	38	197	547
16	36	241	433
17	46	82	179
18	42	110	134
13	38	98	49

the sterol at physiological concentrations of $25(\text{OH})\text{D}_3$. However, extrarenal production can correct serum calcitriol after $25(\text{OH})\text{D}_3$ supplementation [1]. Our laboratory has been interested in defining the location and regulation of the extrarenal sources of calcitriol in chronic uremia. We have reported that peripheral blood mononuclear phagocytes from normal volunteers constitutively express 1α -hydroxylase activity, and that the activity of the enzyme is enhanced in patients undergoing hemodialysis [21]. However, the K_m of the enzyme of normal macrophages is similar to that of the renal enzyme. Thus, unless the K_m for $25(\text{OH})\text{D}_3$ of the enzyme in macrophages is altered in chronic uremia, it is not clear why supraphysiological levels of substrate are required to normalize serum calcitriol in anephric patients. The amount of blood required to examine the K_m of macrophage 1α -hydroxylase has been a limiting factor to further characterize this issue in patients with CRF and has led us to a search for an appropriate experimental model.

The report of hepatic 1α -hydroxylase activity in pigs with an affinity for $25(\text{OH})\text{D}_3$ significantly lower than that of the renal enzyme [12] prompted us to examine the rat liver as a site of extrarenal synthesis of $1,25(\text{OH})_2\text{D}_3$.

We found that rat hepatic tissue can convert $25(\text{OH})\text{D}_3$ to a metabolite that, based on chromatographic properties and on its ability to bind the calf thymus vitamin D receptor, appears to be $1,25(\text{OH})_2\text{D}_3$. We used the free radical scavengers, DPED and EDTA [22], in the incubation media, which suggests that calcitriol production by hepatic tissue is not mediated by free radical generation. Total inhibition of the rat liver enzyme by ketoconazole, a cytochrome P450 inhibitor that interacts with the heme iron of the cytochrome [23], suggests that the rat hepatic 1α -hydroxylase, similar to the renal enzyme, is a cytochrome P450 mixed function oxidase.

Total inhibition of $1,25(\text{OH})_2\text{D}_3$ production in mitochondrial and microsomal subcellular fractions of pig liver with $2 \mu\text{M}$ ketoconazole was reported by Hollis [12]. The requirement for higher amounts of ketoconazole in our experimental conditions can be attributed to (1) less access of ketoconazole to the 1α -hydroxylase in homogenates than to pure subcellular organelles or (2) difficulties in obtaining a homogeneous distribution of ketoconazole in the wells compared to the culture tubes used by Hollis.

Kinetic analysis of the 1α -hydroxylase of rat liver showed a K_m of $16.6 \pm 0.7 \mu\text{M}$ similar to that reported for the enzyme in pig liver. However, there was a marked discrepancy between the V_{max} obtained by us and that reported by Hollis. This could be due to species differences (rat vs. pig) or to the use of

different subcellular fractions (CFH vs. mitochondria). While Hollis showed no saturability of the enzyme with increasing substrate concentrations in the microsomal fraction, in our studies, enzyme saturability was reached in every liver CFH examined. Assessment of the individual contribution of different subcellular fractions in the rat will require further studies.

The lower affinity of the hepatic enzyme for $25(\text{OH})\text{D}_3$ could represent an appealing explanation for the need of increased $25(\text{OH})\text{D}_3$ levels to normalize serum $1,25(\text{OH})_2\text{D}_3$ concentrations in the absence of renal mass. We next examined whether chronic uremia further affects the affinity for substrate of the hepatic 1α -hydroxylase. In uremic rats, the K_m of the hepatic enzyme was significantly higher than in normal animals without significant differences in V_{max} between the two groups. It is possible that uremia could affect in a different manner the expression of the 1α -hydroxylase and/or the accessibility of substrate to each subcellular fraction. Further examination of individual organelle preparations is required to determine if the increased K_m in uremic livers is the result of altered kinetics in the mitochondrial and/or microsomal fractions. The observed difference in K_m between the two groups of animals cannot be attributed to differences in the binding of $25(\text{OH})\text{D}_3$ to DBP since the specific binding of $25(\text{OH})\text{D}_3$ to CFH was not different between normal and uremic rats. Serum level of DBP has been reported to remain constant in patients with varying degrees of renal failure [24], and our *in vitro* assessment of DBP agrees with those findings. It is important to mention that a 10-fold variation in V_{max} was observed in uremic rats. A similar range of V_{max} values (40-fold variation) was reported for the 1α -hydroxylase of pulmonary alveolar macrophages in patients with sarcoidosis [25]. Our findings of increased K_m in uremic rats with no significant modification of V_{max} suggest a competitive inhibition of the hepatic 1α -hydroxylase. These observations agree with Hsu and collaborators [26, 27] that several uremic toxins can suppress the renal $1,25(\text{OH})_2\text{D}_3$ production *in vivo* and *in vitro*. Extrapolation of the uremic rat liver hydroxylase findings to our previous observation in anephric humans suggests that the accumulation of "uremic toxins" during the progression of renal failure may impair the access of $25(\text{OH})\text{D}_3$ to the active site of the extrarenal enzyme, thus requiring higher levels of $25(\text{OH})\text{D}_3$ to overcome the inhibition and restore normal $1,25(\text{OH})_2\text{D}_3$ levels in circulation.

Further studies are required for a precise location of the cell type (hepatocytes, Kupffer cells, endothelial cells or lipocytes) responsible for calcitriol generation by the liver. Modulation of the liver enzyme could be an important therapeutic tool to correct the abnormal vitamin D metabolism of chronic uremia.

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