# 25-HYDROXYCHOLECALCIFEROL: HIGH AFFINITY SUBSTRATE FOR HEPATIC CYTOCHROME P-450

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## SUMMARY

Addition of 25-hydroxycholecalciferol (25-OH-$D_3$) to rat liver microsomes revealed a type I spectral change with a spectral dissociation constant ($K_s$) of approximately 80nM; this value remained unchanged in phenobarbital-treated rats, although the maximum binding value was tripled. The inhibition constant, $K_i$, of 25-OH-$D_3$ for aminopyrine N-demethylation was 59nM, in good agreement with the $K_s$ value. Stopped-flow studies showed a two-fold increase in microsomal NADPH cytochrome P-450 reductase activity in the presence of 25-OH-$D_3$. These findings suggest that the P-450 system plays a role in the biotransformation of 25-OH-$D_3$.

## INTRODUCTION

There is now ample evidence (1-5) that cholecalciferol (vitamin $D_3$) is transformed in the liver to a more polar hydroxylated metabolite, 25-hydroxycholecalciferol (25-OH-$D_3$), which in turn is transformed further in both liver (6) and kidney (7,8). The 25-hydroxylation is thought to occur in the microsomal fraction of the liver, requires oxygen and reduced nicotinamide adenine dinucleotide phosphate (9), but the reaction is said not to be blocked by carbon monoxide, to be unaffected by inhibitors of cytochrome P-450 and not to be induced by phenobarbital (10). Studies in human subjects on chronic phenobarbital therapy have shown that the transformation of vitamin $D_3$ to 25-OH-$D_3$ and other polar metabolites was accelerated (6). In addition, phenobarbital treatment of rats and guinea pigs resulted in increased conversion of vitamin $D_3$ to 25-OH-$D_3$ and to a greater biliary excretion of the more polar and conjugated metab-

olites (11). Moreover, when rats were treated with phenobarbital, there was significant increase in the amount of polar metabolites of vitamin $D_3$ found associated with the smooth endoplasmic reticulum of the liver (12). However, patients chronically treated with phenobarbital and dilantin had lower serum levels of 25-OH-$D_3$ (13).

From the preceding reports it is not clear whether phenobarbital induced a microsomal mixed function oxidase that enhances the transformation of vitamin $D_3$ to 25-OH-$D_3$, or whether the phenobarbital-induced enzyme acts on a subsequent step.

It, therefore, seemed important to determine whether the cytochrome P-450 terminal oxidase system, found in rat liver microsomes, interacts with vitamin $D_3$ and its metabolites to form an enzyme-substrate complex. Such a complex (substrate binding to the oxidized form of cytochrome P-450) elicits a type I spectral change (14) which is attributed to a substrate-induced absorbance shift from 420nm to 385-390nm (15). In the difference spectrum this shift is manifested as a peak at 385-390nm and as a trough at about 420nm. This spectral change reflects the formation of a metabolically active cytochrome P-450-substrate complex. Such shifts have also been observed with a solubilized, as well as a partially purified microsomal cytochrome P-450 system (16,17) and with a homogeneous enzyme preparation from Pseudomonas putida (18).

## METHODS

Male Sprague-Dawley rats (200-250g), purchased from Charles River, Wilmington, Mass., were decapitated and their livers perfused with isotonic saline. The tissue was homogenized in 0.25M sucrose containing 1.0mM Tris-HCl buffer, pH 7.4; microsomes were obtained by differential ultracentrifugation (19). Cytochrome P-450-substrate binding was measured by difference spectroscopy with an Aminco DW-2 UV/VIS spectrophotometer. Cytochrome P-450 reductase was measured at 23°C with the Aminco-Morrow stopped-flow accessory. One syringe of the stopped flow apparatus contained 6mM glucose, 40µl glucose oxidase (Sigma, Type V, 1400 units/ml), 10µl catalase (22,000 Sigma units/ml), microsomes (3mg/ml) and 50mM Tris-HCl, pH 7.4, in a final volume of 6ml gassed with carbon monoxide; a 1.0mM NADPH solution containing the glucose oxidase-catalase system and gassed with carbon monoxide was placed in the other syringe. Aminopyrine N-demethylase activity was determined by measuring formaldehyde (20).

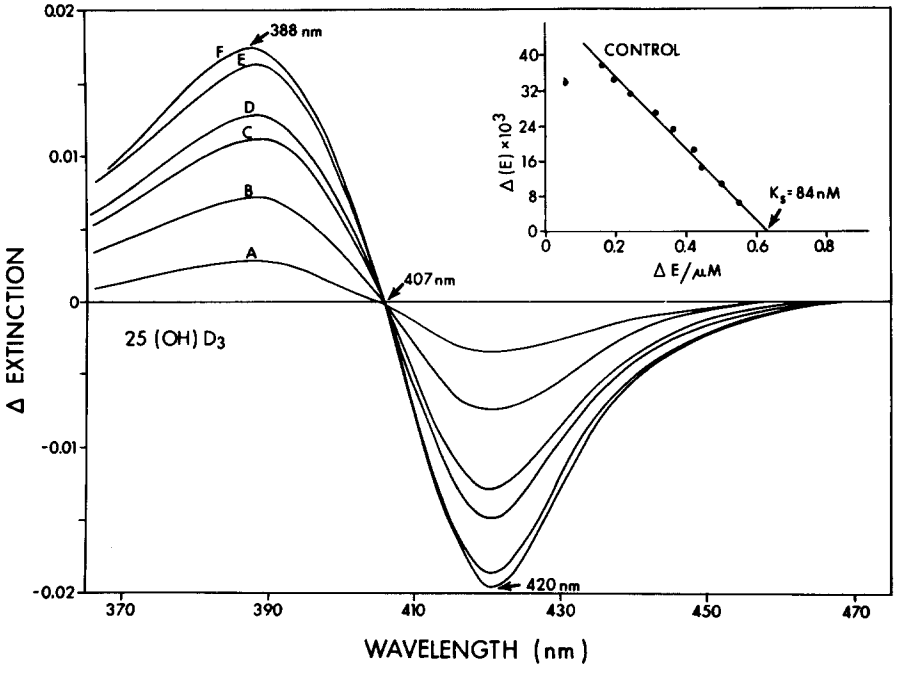


Figure 1. 25-Hydroxycholecalciferol-induced type I spectral change. Liver microsomes from adult rats (2mg protein/ml) in 0.1 M Tris-HCl, pH 7.4) were distributed between two cuvettes; 25-hydroxycholecalciferol was added to the sample and an equivalent volume of buffer to the reference cuvette. Final concentrations of the 25-OH-$D_3$ in a 3ml suspension: A. 11nM, B. 33nM, C. 66nM, D. 88nM, E. 176nM and F. 242nM. Insert: Eadie-Hofstee plot of ΔE 388nM-420nM and concentration of 25-OH-$D_3$.

## RESULTS AND DISCUSSION

As reported by Hahn et al. (6,12) in the presence of NADPH and $O_2$, liver microsomes from untreated and phenobarbital-treated rats metabolized 25-OH-$D_3$ to as yet unidentified more polar products; the rate of metabolism of 25-OH-$D_3$ was 1.5 pmols per min per mg microsomal protein. Figure 1 shows the type I spectral change obtained by the addition of increasing amounts of 25-OH-$D_3$ to liver microsomes from untreated rats. An absorption peak was observed at 388nm, a trough at 420nm and an isosbestic point at 407nm. Analysis of these data by an Eadie-Hofstee plot revealed a spectral dissociation constant ($K_s$) of 84nM, (Figure 1, insert). The $K_s$ for 25-OH-$D_3$ obtained on microsomes from rats given 70mg phenobarbital/

TABLE 1

HALF-MAXIMAL SPECTRAL CHANGES ($K_s$)
AND MAXIMAL BINDING CAPACITY ($B_{max}$)
OF VITAMIN D CONGENERS

| Type I Substrate | $K_S$, nM | $B_{max}$ ($\Delta$E/mg protein) |
|---|---|---|
| Dihydroxytachysterol ($DHT_2$) | 13,500 | ----- |
| Ergocalciferol | 7,100 | ----- |
| 25-hydroxycholecalciferol | 84 | 0.052 |
| 25-hydroxycholecalciferol (phenobarbital treatment) | 84 | 0.163 |

Values were derived from Eadie -Hofstee plots (see text)

Phenobarbital treatment consisted of 70 mg/kg BW, administered intraperitoneally for three successive days.

Protein concentration in liver microsome preparations: 2 mg/ml

kg body weight for 3 days was the same (Table 1), but the maximum binding value in that preparation was tripled. Thus 25-OH-$D_3$, a natural substrate, has the lowest $K_s$ value reported to date. In fact, most substrates, whether natural or xenobiotic, have $K_s$ values 2-3 orders of magnitude greater (14,21,22). As can be seen from Table 1, other vitamin D congeners also served as substrates for the cytochrome P-450 system, although their affinity was lower than that for 25-OH-$D_3$. The order of affinity constants of these compounds bears no obvious relationship to their biological activity. However, the findings can be interpreted as support for the conclusion (23) that lipid solubility does not play a major role in determining the affinity of the enzyme-substrate interaction.

Aminopyrine N-demethylation, a cytochrome P-450-catalyzed oxidative reaction, was competitively inhibited in the presence of 25-OH-$D_3$ (figure 2); the Km for aminopyrine was about 0.4mM in the absence of the 25-OH-$D_3$,

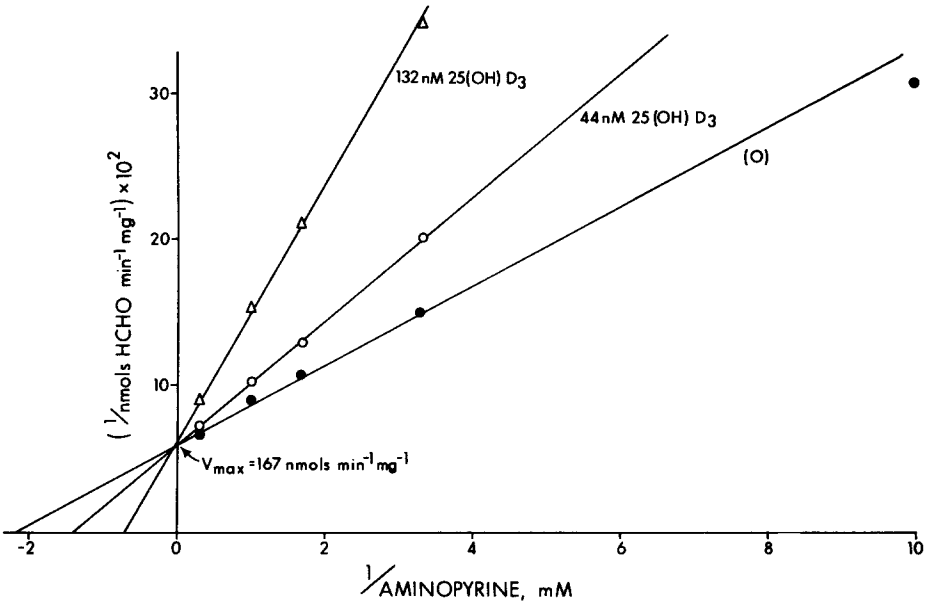


Figure 2. Effect of 25-OH-$D_3$ addition on rat liver microsomal aminopyrine N-demethylation. The Lineweaver-Burk plot of enzyme activity was obtained with 44nM, 132nM or no 25-OH-$D_3$. Both inhibitor and substrate were present in the assay medium which was preincubated for 10 min. at 37° to generate NADPH (from 0.5mM $NADP^+$). The microsomes (1mg/ml final concentration) were added to the medium and incubation continued for 5 min. at 37°C; aminopyrine concentration ranged from 0.1mM to 3mM. The Km and $V_{max}$ values were obtained by the method of least squares.

but was increased to 0.7 and 1.4mM in the presence of 44nM and 132nM 25-OH-$D_3$, respectively. This and the above results indicate that the 25-hydroxy metabolite is an alternate substrate for cytochrome P-450. The calculated inhibition constant, $K_i$, of 25-OH-$D_3$ for aminopyrine-N-demethylation was 59nM, in good agreement with the $K_s$ value of 84 (Table 1). Ergocalciferol and dihydroxytachysterol ($DHT_2$) also inhibited aminopyrine N-demethylation (figure 3), although higher concentrations were required relative to 25-OH-$D_3$.

The reduction of cytochrome P-450 by the flavoprotein reductase is the rate limiting step in microsomal mixed function oxidase reactions (24,25). Substrates of the P-450 system exert a positive modifier ef-

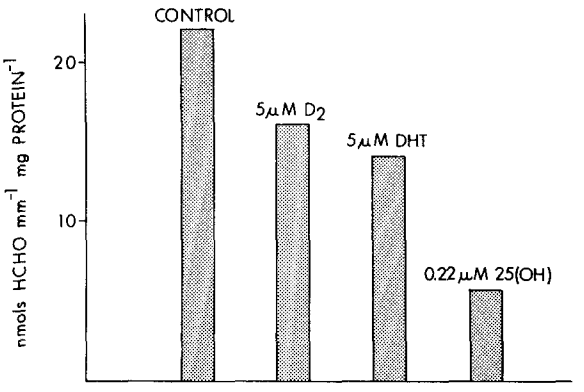


Figure 3. Effect of various vitamin D congeners on aminopyrine N-demethylation by rat liver microsomes. The aminopyrine concentration was 3mM; the inhibitors were ergocalciferol ($D_2$) - 5μM: dihydrotachysterol ($DHT_2$) - 5μM; 25-(OH)-$D_3$ - 0.22μM.

TABLE 2

25-HYDROXYCHOLECALCIFEROL EFFECT ON NADPH CYTOCHROME P-450 REDUCTASE ACTIVITY IN RAT LIVER MICROSOMES

| 25-(OH)-$D_3$ | NADPH P-450 reductase activity nmoles/min/mg microsomal protein Exp. 1 | Exp. 2 |
|---|---|---|
| None | 20 | 18 |
| 100nM | 58 | 50 |

The fixed wavelengths were 450nm-490nm

The reductase activity was measured with the Aminco-Morrow stop-flow apparatus (see text).

fect on this step; for example, NADPH cytochrome P-450 reductase activity is increased in the presence of substrate. Table 2 shows that a similar result was obtained by the addition of 25-OH-$D_3$ which caused the reductase activity to double.

Taken together, these findings -- microsomal metabolism of 25-OH-$D_3$, a very low $K_s$, competitive inhibition of aminopyrine-N-demethylation and a doubling of the cytochrome P-450 reductase activity -- suggest that the P-450 system plays a role in the degradation of vitamin D and its metabolites. This argument is strengthened by the fact that phenobarbital induces the P-450 mixed function oxidase system, resulting in increased binding of 25-OH-$D_3$ to cytochrome P-450 (Table 1). Moreover, chronic phenobarbital treatment of epileptic children has led to signs of vitamin D deficiency (26) and increased formation of more polar metabolites of vitamin D (11). It, therefore, seems logical to suggest that 25-OH-$D_3$ biotransformation is catalyzed by the P-450 system.

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