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Characteristics of the Rat Liver Microsomal Enzyme System Converting Cholecalciferol into 25-Hydroxycholecalciferol

EVIDENCE FOR THE PARTICIPATION OF CYTOCHROME *P*-450

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Properties of the rat hepatic cholecalciferol 25-hydroxylase have been studied. An assay system has been developed in which 25-hydroxycholecalciferol production is linear for at least 2h in both homogenates and microsomal fraction. Furthermore, the initial reaction velocity is linearly related to the amount of liver tissue or microsomal fraction. This enzyme system also metabolizes an analogue of cholecalciferol, namely dihydrotachysterol$_3$, into 25-hydroxydihydrotachysterol$_3$. The 25-hydroxylase is in the microsomal fraction and not in mitochondria. It has a K_m of 44nM for cholecalciferol and 360nM for dihydrotachysterol$_3$. Its activity is not altered by dietary concentrations of calcium and phosphorus. Vitamin D-deficient rats have higher activities of the hepatic 25-hydroxylase than those receiving 25ng of cholecalciferol daily. The 25-hydroxylase is inhibited by metyrapone. An atmosphere of CO/O_2 (9:1, v/v) inhibits the reaction by 87%. This inhibition is partially reversed by white light. Additionally, cholecalciferol and 25-hydroxycholecalciferol competitively inhibit aminopyrine demethylase. These results support the idea that the cholecalciferol 25-hydroxylase is a cytochrome *P*-450-dependent mono-oxygenase.

Previous reports have established that cholecalciferol must be metabolically activated to 25(OH)D_3 in the liver and subsequently in the kidney to 1,25-(OH)$_2D_3$ before it can manifest its physiological functions (DeLuca, 1974; Kodicek, 1974; DeLuca & Schnoes, 1976). Thus the 25-hydroxylase is an important enzyme in the activation of cholecalciferol. Little is known concerning the properties of this enzyme, mostly because minimal activity is exhibited when the enzyme is assayed *in vitro*. Horsting & DeLuca (1969) first reported the ability of rat liver homogenates to convert cholecalciferol into 25-(OH)D_3 when supported by added glucose 6-phosphate and Mg^{2+} utilizing the endogenous glucose 6-phosphate dehydrogenase. It was later reported (Bhattacharyya & DeLuca, 1974) that the enzyme is predominantly found in the microsomal fraction of rat liver homogenates and requires the soluble fraction of the cell for maximal activity. The cytosol contains a heat-labile factor that both protects the substrate and stimulates conversion of cholecalciferol into 25(OH)D_3 and may well be analogous to the system described for cholesterol biosynthesis and for bile acid biosynthesis (Ritter & Dempsey, 1971; Scallen *et al.*, 1971; Grabowski *et al.*, 1976). In addition, some 25-hydroxylation was observed in the mitochondrial fraction, especially at high substrate concentration (Bhattacharyya & DeLuca, 1973), as well as production of a more-polar metabolite not identical with 25(OH)D_3. Further studies (Bhattacharyya & DeLuca, 1973; Horsting & DeLuca, 1969) with inhibitors of mixed-function oxidase systems could not detect any involvement of cytochrome *P*-450 in this hydroxylation reaction. Because of the biological requirements of the enzyme for activity, i.e. O_2 and nicotinamide nucleotides, we have pursued a thorough search for possible mechanisms involved in the mixed-function oxidase. Madhok *et al.* (1978) found incorporation of $^{18}O_2$ into the 25-position of cholecalciferol to form 25(^{18}OH)D_3 by using rat liver postmitochondrial supernatant. Bjorkhem & Holmberg (1978), utilizing rat liver mitochondrial fraction alone and with their mass-fragmentographic assay, have suggested that the 25-hydroxylase is cytochrome *P*-450-dependent. Cinti *et al.* (1976) demonstrated that 25(OH)D_3 is an inhibitor of aminopyrine *N*-demethylase, a cytochrome *P*-450-dependent microsomal reaction in the rat liver. These are a few indications of the involvement of cytochrome *P*-450 as a terminal

Abbreviations used: 25(OH)D_3, 25-hydroxycholecalciferol; 1,25(OH)$_2D_3$, 1,25-dihydroxycholecalciferol; h.p.l.c., high-pressure liquid chromatography.

oxidase in the 25-hydroxylation of cholecalciferol by rat liver microsomal fraction.

The purpose of the present paper is to report the properties of this system, to demonstrate its microsomal location and to report substantial evidence of the participation of cytochrome *P*-450 in this hydroxylation.

Experimental

Materials

[3α-^{3}H]Cholecalciferol (sp. radioactivity 33000 d.p.m./pmol, 15Ci/mmol) was prepared in this laboratory by Dr. Herbert Paaren by using the method of S. Yamada, H. F. DeLuca & H. K. Schnoes (unpublished work). 25(OH)[26,27-^{3}H]D_3 (sp. radioactivity 2640d.p.m./pmol) was prepared by the method of Suda *et al.* (1971). [1,2-^{3}H]Dihydrotachysterol$_3$ (sp. radioactivity 919d.p.m./pmol, 0.35 Ci/mmol) was chemically synthesized in our laboratory by Hallick & DeLuca (1971). It was purified before use by passage through a column (1cm×60cm) of Lipidex 5000 with chloroform/methanol/water (2:7:1, by vol.) as the eluting solvent system. A total of 100 fractions of 4.4ml/fraction was collected. The major peak, out of four radioactive peaks, was collected and identified as dihydrotachysterol$_3$ by mass spectrometry and co-chromatography with crystalline dihydrotachysterol$_3$ on h.p.l.c. The 25-hydroxy[1,2-^{3}H]dihydrotachysterol$_3$ was biosynthesized from [1,2-^{3}H]dihydrotachysterol$_3$ (Hallick & DeLuca, 1971). It was further purified before use by passage through a column (1cm×60cm) of Lipidex 5000 with hexane/chloroform (9:1, v/v) as solvent system. Elution was accomplished by using the same solvent system. A total of 50 fractions of 3ml/fraction was obtained and the 25-hydroxydihydrotachysterol$_3$ peak was pooled and used. Crystalline cholecalciferol, 25(OH)D_3 and dihydrotachysterol$_3$ were gifts of the Philips–Duphar Co., Weesp, The Netherlands. Metyrapone was a gift from CIBA–GEIGY Corporation. Aminopyrine was a gift from Dr. Elizabeth Jeffery, Department of Pharmacology, University of Minnesota, Minneapolis, MN, U.S.A. Other chemicals were purchased from Sigma Chemical Co. All solvents used were of analytical grade.

Animals

Unless otherwise stated, male weanling rats (Holtzman Co., Madison, WI, U.S.A.) were maintained in hanging wire cages and fed *ad libitum* a vitamin D-deficient 0.02% calcium diet for 3 weeks (Suda *et al.*, 1970).

Preparation of subcellular fractions

The rats were killed by cervical dislocation, and the livers were immediately removed and carefully separated from the adhering connective tissue. The livers were rinsed with ice-cold 0.25M-sucrose, transferred to an ice-cold Potter–Elvehjem homogenizer fitted with a Teflon pestle, and homogenized in 3vol. of the sucrose. The homogenate was centrifuged at 800*g* at 4°C for 10min to sediment a crude nuclear pellet by using a Lourdes centrifuge equipped with a 9 RA rotor. The resulting supernatant was centrifuged for 15min at 10000*g* to sediment most of the mitochondrial fraction. In experiments where a pure mitochondrial fraction was needed, the method of DeLuca *et al.* (1960) was employed. As judged by glucose 6-phosphatase assay, the mitochondrial fraction contained less than 7% microsomal contamination. The postmitochondrial supernatant was finally centrifuged at105000*g* for 60min in a Beckman L5-50 ultracentrifuge equipped with a Ti50 rotor to produce a microsomal pellet. The microsomal pellet was resuspended in 0.25M-sucrose to give a final concentration of 20mg of protein/ml as determined by the procedure of Lowry *et al.* (1951) with bovine serum albumin as standard. As judged by succinate dehydrogenase assay, the microsomal suspension is completely free of mitochondrial contamination. The upper fatty layer of the cytosolic fraction was removed with a Pasteur pipette and the reddish supernatant remaining was used.

Incubation conditions and extraction of samples

Unless otherwise stated, a typical incubation mixture contained the following (Bhattacharyya & DeLuca, 1974). To a 125ml Erlenmeyer flask was added 2.5ml of cell fraction suspension, 2.5ml of cytoplasmic fraction (or simply 4–5ml of liver homogenate), 2.5ml of buffer cofactor solution (0.1M-potassium phosphate buffer, 0.4mM-$NADP^+$, 160mM-nicotinamide, 20mM-ATP and 22.4mM-glucose 6-phosphate adjusted to pH7.4), 2.5ml of salt solution (5mM-$MgCl_2$, 0.1M-KCl and 0.25 unit of glucose 6-phosphate dehydrogenase/ml as defined by Sigma Chemical Co., St. Louis, MO, U.S.A.) and enough 0.25M-sucrose to make a total volume of 10ml. Each flask was gassed with 100% O_2 for 1min. Then [3α-^{3}H]cholecalciferol (425pmol, 2.5×10^5d.p.m.) was added to each flask in 10μl of 95% ethanol. Incubation was carried out at 120 oscillations/min for 2h (with microsomal fraction) or 4h (with liver homogenate) at 37°C. Reactions were terminated by addition of 40ml of methanol/chloroform (2:1, v/v). Each mixture was transferred to a 125ml separatory funnel. Each flask was rinsed with 20ml of chloroform. The chloroform rinse was combined with the rest of the mixture in the funnel and shaken. The mixture was left overnight at 4°C to allow complete separation of the chloroform layer from the aqueous layer. The chloroform layer was filtered through a shark-skin filter paper into a 250ml round-bottom flask. The mixture was re-extracted once again with

an additional 20ml of chloroform. The chloroform layers were combined and evaporated to dryness by using a rotary evaporator at 40°C. The residue was transferred quantitatively to a 10ml volumetric flask by using hexane/chloroform (9:1, v/v) and 50μl was taken for radioactivity determination. The combined chloroform extracts always contained 100% of the added radioactivity. When radioactive 25(OH)D_3 was incubated for 4h under these conditions, it was not degraded, giving 80–90% recovery on chromatography.

Chromatography

All lipid extracts were applied to columns (1.5cm × 20cm) of Lipidex 5000 in hexane/chloroform (9:1, v/v). Elution was accomplished with the same solvent system, collecting a total of sixty 3ml fractions. The solvents were evaporated under a stream of air and the resulting residues were redissolved in 4ml of toluene solution [2g of 2,5-diphenyloxazole and 100mg of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/litre of toluene] for counting radioactivity. The ^3H in the vials was determined with a Packard Tri-Carb model 3375 liquid-scintillation counter. A typical chromatogram from an incubation mixture is shown (Fig. 1). The 25-hydroxydihydrotachysterol$_3$ and dihydrotachysterol$_3$ assume the same positions with this chromatographic system as 25(OH)D_3 and cholecalciferol respectively. After each group of determinations, the columns were stripped with 50ml of hexane/chloroform (4:1, v/v). They were regenerated by washing with 50ml of hexane/chloroform (9:1, v/v) before applying a new sample. A column can be used several times before it must be discarded. The identity of the product was positively identified by co-chromatography with authentic 25(OH)D_3 or dihydrotachysterol$_3$ on h.p.l.c. as described by Jones & DeLuca (1975). No other metabolites were observed in these studies.

Inhibition of 25-hydroxylase by CO and reversal of inhibition by white light

Gases like CO and N_2 were made free of contaminating O_2 by passage through a solution of 10% pyrogallol in 5% KOH. Traces of the alkali were removed by passage of the deoxygenated gases through 19mM-$Na_2S_2O_3$ and 50mM-Tris/HCl, pH 8.0. Purified gases were finally stored in reservoirs fitted with rubber septa. Gases were removed from the reservoirs by means of a gas-tight syringe. Appropriate amounts of individual gases were immediately emptied into evacuated 15ml serum bottles containing the usual incubation mixtures. Hydroxylation reactions were initiated by introduction of [3α-^3H]-cholecalciferol in 10μl of 95% ethanol by using a Hamilton syringe.

For the experiment on light reversal of CO inhibition, a high-intensity 750 W Bausch and Lomb projection lamp (with intensity of 77.5klx as measured by a Gossen light-meter) equipped with focusing lens was used as the source of white light. The light was allowed to pass through a thin-glass window of a polyacrylic temperature-regulated water bath, and was reflected into the vessel by a mirror. The incubation mixture in a 25ml Erlenmeyer flask was illuminated, therefore, from the bottom.

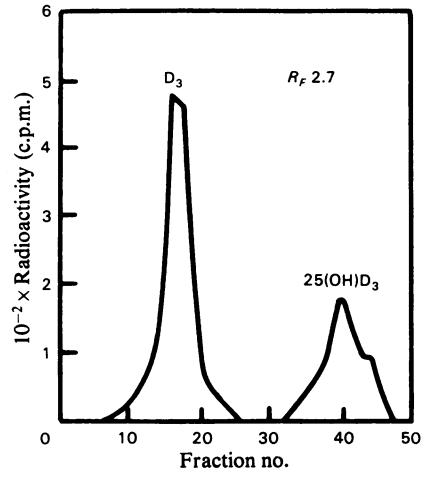

Fig. 1. *Separation of cholecalciferol and 25(OH)D_3 by using Lipidex 5000*

A Lipidex 5000 (hydroxyalkoxypropyl derivative of Sephadex LH-20; Packard Instrument Co. Inc., Des Plains, IL, U.S.A.) column (1.5cm × 20cm) was prepared with hexane/chloroform (9:1, v/v) as solvent. Extracted sterols from a 25-hydroxylase assay system were loaded on the column. Elution was accomplished with hexane/chloroform (9:1, v/v). Fractions (3ml) were collected from the column and the fractions were analysed for radioactivity. Cholecalciferol (D_3) (tubes 11–22) was eluted first followed by 25(OH)D_3 (tubes 32–47). Recovery of added radioactivity was 100%.

Assay of microsomal aminopyrine N-demethylase activity

The assay is an adaptation of the procedure of Sladek & Mannering (1969*a*). Formaldehyde that was liberated by oxidative *N*-demethylation of aminopyrine was determined by the method of Nash (1953).

Results

Effect of time, amount of liver tissue and various concentrations of cholecalciferol on the rat liver homogenate conversion of cholecalciferol into 25(OH)D_3

With whole homogenates, the conversion of cholecalciferol into 25(OH)D_3 in the above described

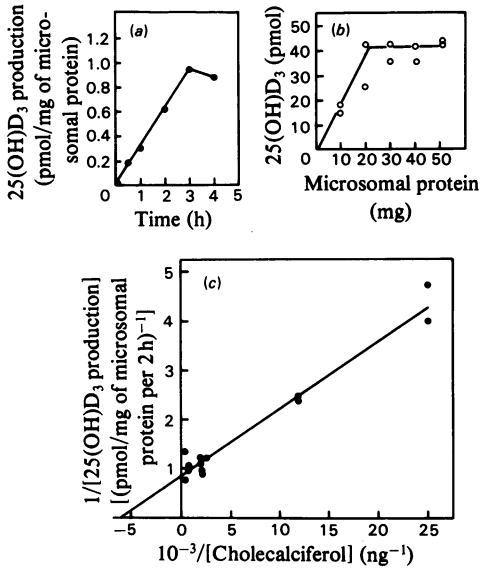

Fig. 2. *Characteristics of the hepatic microsomal cholecalciferol 25-hydroxylase*

(*a*) Time course of the microsomal conversion of cholecalciferol into 25(OH)D_3. The incubation mixture contained 18.7mg of microsomal protein, 47.5mg of cytosol protein and the ingredients described in the Experimental section. Incubations were carried out for 0, 0.5, 1, 2 and 3h at 37°C. (*b*) Effect of various amounts of rat liver microsomal protein on 25-hydroxylase activity. Various amounts of rat liver microsomal protein from 0 to 51mg were used in the incubation. (*c*) K_m measurement for the microsomal conversion of cholecalciferol into 25(OH)D_3. The incubation mixture contained 20.8mg of microsomal protein, 56.5mg of cytosol protein and the ingredients described in the Experimental section. Various concentrations of cholecalciferol from 0.125mmol to 6.53nmol (sp. radioactivity of 992000d.p.m./nmol to 19100d.p.m./nmol respectively) were added to 10μl of 95% ethanol. The K_m is calculated by the method of least squares. The remaining incubation conditions were as described in the Experimental section.

system was linear up to 5h. Enzyme activity was also linearly related up to 1g of liver tissue. The K_m for the rat liver homogenate enzyme system was calculated as 60nM by the method of least squares.

Effect of time and various concentrations of cholecalciferol on the rat liver microsomal conversion of cholecalciferol into 25(OH)D_3

The rate of rat liver microsomal conversion of cholecalciferol into 25(OH)D_3 was linear up to 3h of incubation, beyond which time enzyme activity declined (Fig. 2*a*). Enzyme activity was also linear with up to 20mg of microsomal protein (Fig. 2*b*). The K_m for the rat liver microsomal conversion of cholecalciferol into 25(OH)D_3 was 44nM as calculated by the method of least squares (Fig. 2*c*).

Comparison of the 25-hydroxylation of cholecalciferol and dihydrotachysterol$_3$ by liver subcellular fractions from vitamin D-deficient and vitamin D-supplemented rats

The K_m for the rat liver microsomal conversion of dihydrotachysterol$_3$ into 25-hydroxydihydrotachysterol$_3$ was 360nM, which is 8-fold greater than that found for cholecalciferol (Fig. 2*c*). We observed that in both vitamin D-deficient and vitamin D-supplemented rats, 25-hydroxylase activity for cholecalciferol (Table 1) and dihydrotachysterol$_3$ is located in the microsomal fraction, requiring for full activity the soluble fraction of the homogenate in addition to O_2 and NADPH. As found before (Bhattacharyya & DeLuca, 1973, 1974), microsomal conversion of cholecalciferol into 25(OH)D_3 was decreased to almost half the activity on supplementation of vitamin D-deficient rats with 1 unit of cholecalciferol/day for 3 weeks (Table 2). Enzyme activity responsible for converting dihydrotachysterol$_3$ into 25-hydroxydihydrotachysterol$_3$ on the other hand is not affected by cholecalciferol supplementation of rats. The product of microsomal hydroxylation of cholecalciferol was confirmed as 25(OH)D_3 by h.p.l.c. (Fig. 3). Similarly the product obtained from mitochondrial

Table 1. *Subcellular localization of the hepatic enzyme system metabolizing cholecalciferol and dihydrotachysterol$_3$ to their 25-hydroxy derivatives in vitamin D-deficient and vitamin D-supplemented rats*

This experiment is representative of six such experiments that gave identical results.

Subcellular fractions	25-Hydroxy derivatives synthesized (pmol/ng of protein per 2h)	
	−Vitamin D	+Vitamin D
Cholecalciferol		
Mitochondria+cytosol	<0.01	<0.01
Microsomal fraction+cytosol	0.38	0.24
Dihydrotachysterol$_3$		
Mitochondria+cytosol	<0.04	<0.04
Microsomal fraction+cytosol	1.70	1.80

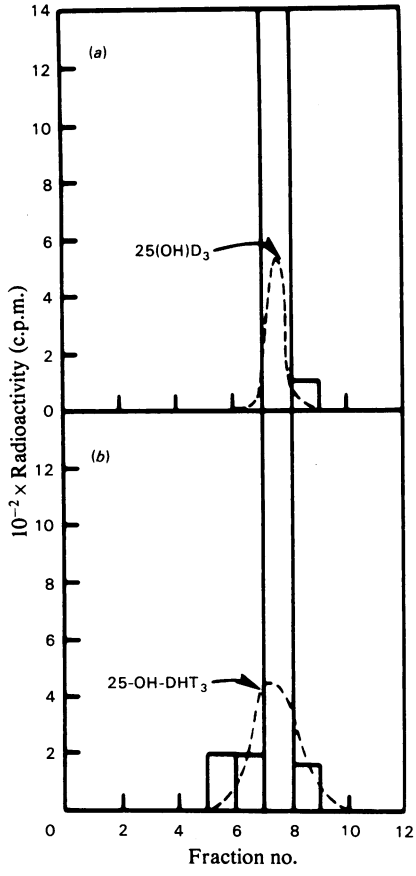

Fig. 3. *Identification of the metabolite produced on incubation of cholecalciferol and dihydrotachysterol$_3$ with the rat liver microsomal system by h.p.l.c.*

The metabolites produced on microsomal incubation of cholecalciferol and dihydrotachysterol$_3$ were pooled, dried under N_2, dissolved in 20μl of 4% propan-2-ol/hexane and injected into an h.p.l.c. apparatus by using a Zorbax-SIL column (0.46cm × 25cm) (Dupont, Wilmington, DE, U.S.A.) for cholecalciferol and a Partisil X-10 column (0.45cm × 50cm) (Waters Associates, Milford, MA, U.S.A.) for dihydrotachysterol$_3$ (25-OH-DHT$_3$). In either case elution was accomplished with 4% propan-2-ol/hexane, collecting 2.4ml/fraction at 6900kPa. The 25(OH)D$_3$ arising from cholecalciferol incubations (*a*) was identified by its co-chromatography with authentic 25(OH)D$_3$, whereas the 25-hydroxydihydrotachysterol$_3$ arising from dihydrotachysterol$_3$ incubations (*b*) was identified by making a separate run of authentic radioactive 25-hydroxydihydrotachysterol$_3$ and noting the position occupied compared with the incubation product.

incubations was also 25(OH)D$_3$ (Table 1). No other metabolite was observed. Also, only one product, i.e. 25-hydroxydihydrotachysterol$_3$, was observed on incubation of the microsomal fraction with dihydrotachysterol$_3$, as determined by a similar run on h.p.l.c. of authentic 25-hydroxydihydrotachysterol$_3$ (Fig. 3*b*). Any metabolite observed with mitochondrial incubations of dihydrotachysterol$_3$ proved to be 25-hydroxydihydrotachysterol$_3$, and no other metabolite was observed.

Table 2. *Effect of dietary concentrations of vitamin D, calcium (Ca) and phosphorus (P) on the cholecalciferol 25-hydroxylase*

Male weanling rats were fed on the indicated diets for 3 weeks. Where indicated, rats were given 1 unit of cholecalciferol daily. Incubation mixtures contained 5.0ml of homogenate (1g of liver) as described in the text. The reaction was allowed to proceed for 4h at 37°C under an O_2 atmosphere. Reactions were stopped and metabolites were analysed as before. Numbers in parentheses represent the numbers of rats per group. Results are means ± S.E.M.

Diet composition	25(OH)D$_3$ (pmol/g of tissue per 4h)
1.2% Ca, 1.2% P+cholecalciferol	29 ± 2.6 (6)
1.2% Ca, 0.1% P	41.8 ± 6.9 (6)
0.47% Ca, 0.3% P	56.3 ± 7.4 (5)
1.2% Ca, 0.3% P	59 ± 4.4 (6)
0.02% Ca, 0.3% P	60 ± 4.2 (6)
0.47% Ca, 0.3% P	45.3 ± 7.1 (4)
0.47% Ca, 0.3% P+cholecalciferol	30.5 (3)
0.02% Ca, 0.3% P	42.8 ± 6.1 (5)
0.02% Ca, 0.3% P+cholecalciferol	31.5 ± 5.7 (5)

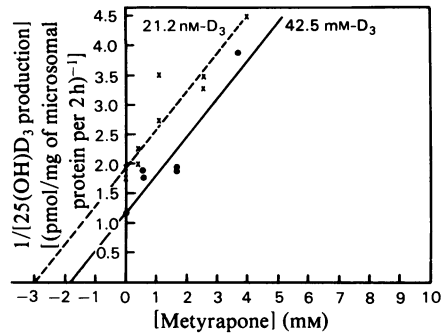

Fig. 4. *Inhibition of 25-hydroxylase by various concentrations of metyrapone*

Various concentrations from 0.5mM to 5mM of metyrapone were added to the incubation vessels. Two separate experiments were conducted by using either 21.2nM-cholecalciferol (D$_3$) or 42.5nM-cholecalciferol. Microsomal protein (20mg) and cytosol (52.5mg of protein) were added to each incubation mixture. The incubation mixtures were as described in the Experimental section.

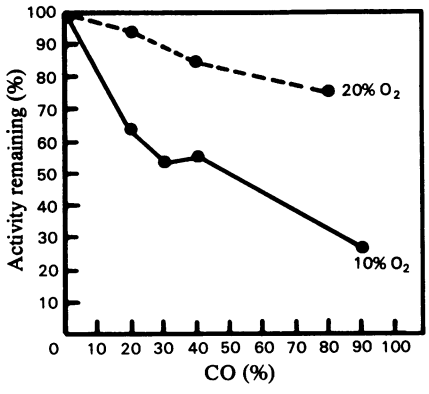

Fig. 5. *Kinetics of the CO inhibition of 25-hydroxylase*
Two sets of experiments were conducted, one where 10% O_2 was used, and the other where 20% O_2 was used, and the percentages of CO were 20, 30, 40 and 90%. Purified N_2 was used to provide the remaining gas. Each reaction mixture contained 20.7mg of microsomal protein, 69mg of cytosol protein and the components described in the Experimental section.

Possible dietary influence on cholecalciferol 25-hydroxylase activity

Male weanling rats were given various laboratory diets, that is: (*a*) a vitamin D-supplemented diet designed by Steenbock (1923); (*b*) 1.2% calcium, 0.1% phosphorus diet (low-phosphorus diet); (*c*) 0.47% calcium, 0.3% phosphorus diet (normal calcium and phosphorus diet); (*d*) 1.2% calcium, 0.3% phosphorus diet (high-calcium diet); (*e*) 0.02% calcium and 0.3% phosphorus diet (low-calcium diet) for 3 weeks. As shown in Table 2, higher enzymic activity is observed in rats on vitamin D-deficient diets compared with that of the rats given a stock diet containing vitamin D. However, no influence of dietary calcium or phosphorus could be detected on the 25-hydroxylase activity. In another experiment, the intraperitoneal injection of 1 unit of cholecalciferol/day to rats fed various amounts of calcium caused an average decrease of 30% in 25-hydroxylase activity compared with that of vitamin D-deficient controls (Table 2).

Table 3. *Light reversal of CO inhibition of 25-hydroxylase*
Each incubation mixture, in 25ml Erlenmeyer flasks, contained 19.4mg of postmitochondrial protein and the rest of the ingredients as described in the Experimental section in a total volume of 2.0ml. Reaction mixtures were put in the necessary gas mixtures and preincubated for 1h at 37°C before irradiation was started. The above experiment was repeated three times with identical results.

Gas system	Exposure to light	25(OH)D_3 (pg/mg of protein per 5h)	Enzyme activity (%)
10% O_2/90% N_2	—	165.56	100
10% O_2/90% CO	—	73.48	44 (32% reversal by light)
10% O_2/90% CO	+	126.64	76

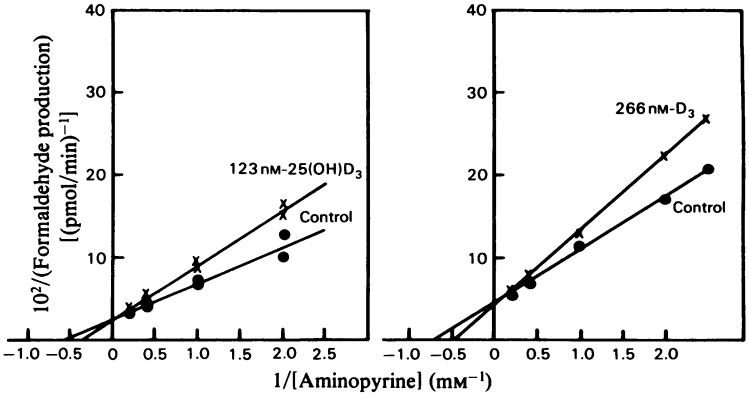

Fig. 6. *Effect of 25(OH)D_3 and cholecalciferol on microsomal aminopyrine N-demethylation*
The procedure is an adaptation of that of Sladek & Mannering (1969*b*). Formaldehyde is measured by the method of Nash (1953). In one experiment, 123nM-25(OH)D_3 in 10μl of ethanol was added to the reaction mixture. In another experiment, 266nM-cholecalciferol (D_3) in 10μl of 95% ethanol was added to the reaction mixture.

Effect of addition in vitro of metyrapone on cholecalciferol 25-hydroxylase

In Fig. 4, various concentrations from 0.5mM to 5mM of metyrapone were added to the reaction medium in parallel experiments with two concentrations of substrate, 21.2nM- or 42.5nM-cholecalciferol. With the use of 42.5nM-cholecalciferol as substrate, K_i of metyrapone was 1.95mM and $V_{max.}$ was 0.8219pmol of 25(OH)D_3 synthesized/mg of protein per 2h, whereas with 21.2nM-cholecalciferol as substrate, K_i of metyrapone was 3.02mM and $V_{max.}$ was 0.5094pmol of 25(OH)D_3 synthesized/mg of protein per 2h. Metyrapone, therefore, is an inhibitor of the cholecalciferol 25-hydroxylase.

Inhibition of rat liver 25-hydroxylase by CO and reversal of inhibition by white light

As shown in Fig. 5, maximum inhibition (87%) of cholecalciferol 25-hydroxylase was observed when incubation was carried out in an atmosphere of O_2/CO (9:1, v/v). Incubations in an atmosphere of decreasing ratios of O_2/CO resulted in a linear decrease in enzyme activity as shown in Fig. 5. Inhibition by CO is reversed by 32% on exposure of the preincubated medium to white light (Table 3).

Effect of administration of phenobarbital on cholecalciferol 25-hydroxylase

Intraperitoneal injection of sodium phenobarbital to vitamin D-deficient calcium-deficient rats produced a slight increase (45%) in the liver 25-hydroxylase activity [control, 24.8±2.35pmol of 25(OH)D_3/g of tissue per 4h; phenobarbital treatment, 36.28± 5.78pmol of 25(OH)D_3/g of tissue per 4h (mean± S.D.)].

Effect of additions in vitro of cholecalciferol on rat liver microsomal aminopyrine N-demethylase activity

As shown in Fig. 6, addition of 123nM-25(OH)D_3 to the aminopyrine *N*-demethylase system increased the apparent K_m of the enzyme from 1.72mM to 2.62mM, i.e. 52% inhibition of enzyme activity, whereas addition of 266nM-cholecalciferol to the enzyme system increased the apparent K_m of the reaction from 1.32mM to 2.12mM, i.e. 61% inhibition of enzyme activity. Inhibition is apparently competitive in nature.

Discussion

To study the properties of the cholecalciferol 25-hydroxylase we developed a whole homogenate system that produces 25(OH)D_3 linearly for 5h and in which reaction rate is linearly related up to 1g of liver tissue. With this system, the K_m for cholecalciferol is 60nM.

There has been some controversy regarding the subcellular localization of the 25-hydroxylase. Contrary to other investigators (Bjorkhem & Holmberg, 1978) we could not detect any succinate-supported mitochondrial activity, nor could we observe significant activity without the cytosolic fraction added to the microsomal fraction. These results agree with and support the previous demonstration that the 25-hydroxylase is microsomal (Bhattacharyya & DeLuca, 1974). Any 25-hydroxylase activity found in the mitochondrial fraction under our conditions is likely to be the result of microsomal contamination. This may account for the results of Bjorkhem & Holmberg (1978). We have not, however, exactly reproduced their experiments. In addition, there appears to be an obligatory requirement for the soluble fraction of the rat liver homogenates for maximal activity to be observed with the microsomal fraction (Bhattacharyya & DeLuca, 1974). Previous work (Bhattacharyya & DeLuca, 1974) has shown that cholecalciferol is easily degraded to unknown substances when cytosol is excluded from the medium. Dihydrotachysterol$_3$, when incubated with subcellular fractions in the absence of cytosol, is also degraded (T. C. Madhok & H. F. DeLuca, unpublished work). It is possible that the liver microsomal lipid-peroxidation system may be responsible for the degradation. This is supported by the observation that *NN'*-diphenyl-*p*-phenylenediamine, a known inhibitor of that system, will block this degradation of cholecalciferol. Bhattacharyya & DeLuca (1974) first found that if cholecalciferol is incubated with microsomal fraction alone, addition of *NN'*-diphenyl-*p*-phenylenediamine prevents destruction of cholecalciferol, the cholecalciferol being recovered unchanged. Thus Bjorkhem & Holmberg (1978) might not have observed reliable microsomal activity without the addition of cytosol. However, we cannot exclude the possibility that saturating amounts of cholecalciferol and a shorter time of incubation, as was adopted by Bjorkhem & Holmberg (1978), might obviate the problem of the destruction of the substrate.

The 25-hydroxylation of dihydrotachysterol$_3$, a reduction product of cholecalciferol known to have cholecalciferol-like biological activity, is apparently not regulated (Bhattacharyya & DeLuca, 1973). It is important to discover whether the same enzyme hydroxylates both cholecalciferol and dihydrotachysterol$_3$. Our results show that the microsomal fraction and not mitochondria are responsible for metabolizing dihydrotachysterol$_3$ to 25-hydroxydihydrotachysterol$_3$. Again, cytosol is an obligatory requirement for full activity. As demonstrated previously (Bhattacharyya & DeLuca, 1973), the administration of vitamin D to rats did not affect the ability of the rat liver microsomal enzyme to convert dihydrotachysterol$_3$ to 25-hydroxydihydrotachysterol$_3$. The K_m of the microsomal enzyme for dihydrotachysterol$_3$ is 360nM, which is about 8-fold higher than that observed with cholecalciferol 25-hydroxylase.

Thus it is uncertain if the same enzyme hydroxylates both substances, but at least the idea that dihydrotachysterol$_3$ is hydroxylated to 25-hydroxydihydrotachysterol$_3$ in mitochondria can be excluded.

Neither dietary calcium nor dietary phosphorus seem to influence cholecalciferol 25-hydroxylase activity. Only feeding of cholecalciferol seems to affect enzyme activity. This phenomenon was discussed fully by Horsting & DeLuca (1969). This suppression of enzyme activity by feeding of cholecalciferol to rats is still the subject of investigation and no firm explanation can be offered at this moment.

Cytochrome *P*-450 plays a major role in most biological hydroxylation reactions as the terminal-oxygen-activating enzyme for such reactions. Mason (1957) introduced the term 'mixed function oxidase' for such enzymes catalysing the introduction of an atom of molecular oxygen into the substrate molecule concomitant with the oxidation of reduced nicotinamide nucleotide. Inasmuch as cholecalciferol 25-hydroxylase requires atmospheric O_2, which is the source of the oxygen for the 25-hydroxy function and NADPH, we have carefully considered the possibility that cytochrome *P*-450 participates in the 25-hydroxylation of cholecalciferol. Our results suggest the enzyme belongs to the mixed-function oxidase class. The evidence for participation of cytochrome *P*-450 in 25-hydroxylation reactions has been derived from (*a*) the inhibition of enzyme activity by CO, (*b*) photoreversibility of the CO inhibition, (*c*) the inhibition by drugs such as metyrapone, (*d*) the inhibition of other cytochrome *P*-450-dependent reactions by cholecalciferol and 25(OH)D$_3$ and (*e*) phenobarbital induction.

CO inhibition of mixed-function oxidase can be attributed to its binding to the cytochrome *P*-450 eliciting the typical absorption band at 450nm (Omura *et al.*, 1965). Such CO inhibition of biological reactions may be reversed by white light. Maximum reversal of CO inhibition is observed when light of 450nm is shone on the incubation mixture. Such a phenomenon was observed with the microsomal cholecalciferol 25-hydroxylase. Maximum inhibition by CO was 87% with a ratio of O_2/CO of 1:9 (v/v). This inhibition by CO was reversed by 32% by white light from a high-intensity projection lamp. Bhattacharyya & DeLuca (1974) failed to observe significant inhibition of the cholecalciferol 25-hydroxylation with 20% O_2 and 80% CO. Only 10% inhibition was observed with their concentration of CO in the present study with 20% O_2, whereas CO caused 87% inhibition when 10% O_2 was present. Therefore Bhattacharyya & DeLuca (1974) failed to observe significant inhibition because of the oxygen tension used. Metyrapone is known to be an inhibitor of both microsomal and mitochondrial hydroxylation reactions (Hildebrandt, 1972; Jefcoate *et al.*, 1973). Experiments revealed a sensitivity of the cholecalciferol 25-hydroxylase system to metyrapone (K_i of 3mM for 21.2nM-cholecalciferol and a K_i of 1.95mM for 42.5nM-cholecalciferol). Another cholecalciferol hydroxylase, chick kidney mitochondrial 25(OH)D$_3$ 1α-hydroxylase, has been extensively studied, purified and also found to be mediated by cytochrome *P*-450 (Ghazarian *et al.*, 1974). Metyrapone and also aminoglutethemide inhibit the 25(OH)D$_3$ 1α-hydroxylase in a competitive manner. These inhibitors had not been tested by Bhattacharyya & DeLuca (1974).

The use of 25(OH)D$_3$ and cholecalciferol as possible inhibitors of the cytochrome *P*-450 microsomal aminopyrine *N*-demethylation reaction was investigated. As was seen by Cinti *et al.* (1976), 25(OH)D$_3$ at a concentration of 123nM caused a shift of apparent K_m for aminopyrine from 1.72mM to 2.62mM, whereas cholecalciferol at 266nM increased the apparent K_m for aminopyrine from 1.32mM to 2.12mM. Thus the cholecalciferol and 25(OH)D$_3$ can be competitive inhibitors of other cytochrome *P*-450-dependent microsomal reactions.

Another line of evidence was the use of the well known inducer of cytochrome *P*-450, phenobarbital. In our previous work, we used the microsomal system together with the cytosol. The chromatogram obtained from phenobarbital-treated samples showed 50% recovery of the radioactive material from the column. Besides, four peaks were obtained: (1) a large non-polar peak in the region of the esters; (2) two peaks of almost equal intensity, one in the region of cholecalciferol and the other appearing as a shoulder on the cholecalciferol peak; (3) a fourth peak in the region of 25(OH)D$_3$. If incubations were done in the presence of *NN'*-diphenyl-*p*-phenylenediamine, the chromatogram appeared similar to the one obtained from control experiment and little increase in activity that could be attributed to phenobarbital was noted (T. C. Madhok & H. F. DeLuca, unpublished work). Several explanations can be offered: (1) phenobarbital induces a microsomal system responsible for the degradation of cholecalciferol that is inhibited by *NN'*-diphenyl-*p*-phenylenediamine; (2) phenobarbital induces further metabolism of 25(OH)D$_3$ to unknown metabolites, either more polar or less polar or both; (3) phenobarbital is affecting the cytosolic protein and not the microsomal protein. We cannot at this time deduce which possibility is correct.

Intraperitoneal administration of phenobarbital to vitamin D-deficient calcium-deficient rats and also normal rats (T. C. Madhok & H. F. DeLuca, unpublished work) produced a slight increase in liver microsomal 25-hydroxylase. This is similar to the findings of Bjorkhem & Holmberg (1978) who demonstrated a 2-fold increase in rat liver 25-hydroxylation of cholecalciferol. Many investigators

have shown that multiple forms of cytochrome *P*-450 exist in the livers of several species. These haemoproteins were distinguished by their immunological response to specific antibodies, their catalytic behaviour, spectral properties and also with various inducers that became the basis for their separation and purification to homogeneity (Sladek & Mannering, 1966, 1969*b*; Alvares *et al.*, 1967, 1968; Hildebrandt *et al.*, 1968; Comai & Gaylor, 1973; Thomas *et al.*, 1976). Therefore it is possible that although phenobarbital behaves as a universal inducer for a multitude of microsomal hydroxylating systems, it may not be the right inducer for the rat liver microsomal 25-hydroxylase (Ernster & Orrenius, 1965). These findings therefore necessitate investigations with other drug inducers to confirm this hypothesis.

The evidence reported in the present paper supports the concept that the microsomal 25-hydroxylation of cholecalciferol is a cytochrome *P*-450-dependent system. These results provide an impetus to attempt solubilization, purification and reconstitution of the components of this system that would provide rigorous proof of the nature of this hydroxylase.

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References

Alvares, A. P., Schilling, G., Levin, W. & Kuntzman, R. (1967) *Biochem. Biophys. Res. Commun.* **24**, 668–674

Alvares, A. P., Schilling, G., Levin, W. & Kuntzman, R. (1968) *J. Pharmacol. Exp. Ther.* **163**, 417–424

Bhattacharyya, M. H. & DeLuca, H. F. (1973) *J. Biol. Chem.* **248**, 2974–2977

Bhattacharyya, M. H. & DeLuca, H. F. (1974) *Arch. Biochem. Biophys.* **160**, 58–62

Bjorkhem, I. & Holmberg, I. (1978) *J. Biol. Chem.* **253**, 842–849

Cinti, D., Golub, E. & Bronner, F. (1976) *Biochem. Biophys. Res. Commun.* **72**, 545–553

Comai, K. & Gaylor, J. L. (1973) *J. Biol. Chem.* **248**, 4947–4955

DeLuca, H. F. (1974) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **33**, 2211–2219

DeLuca, H. F. & Schnoes, H. K. (1976) *Annu. Rev. Biochem.* **45**, 631–666

DeLuca, H. F., Reiser, S., Steenbock, H. & Kaesberg, P. (1960) *Biochim. Biophys. Acta* **40**, 526–530

Ernster, L. & Orrenius, S. (1965) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **24**, 1190–1199

Ghazarian, J., Jefcoate, C. R., Knutson, J. C., Orme-Johnson, W. H. & DeLuca, H. F. (1974) *J. Biol. Chem.* **249**, 3026–3033

Grabowski, G. A., McCoy, K. E., Williams, G. C., Dempsey, M. E. & Hanson, F. R. (1976) *Biochim. Biophys. Acta* **441**, 380–390

Hallick, R. B. & DeLuca, H. F. (1971) *J. Biol. Chem.* **246**, 5733–5738

Hildebrandt, A. G. (1972) in *Biological Hydroxylation Mechanisms* (Boyd, G. S. & Smellie, R. M. S., eds), pp. 79–102, Academic Press, New York and London

Hildebrandt, A., Remmer, H. & Estabrook, R. W. (1968) *Biochem. Biophys. Res. Commun.* **30**, 607–612

Horsting, M. & DeLuca, H. F. (1969) *Biochem. Biophys. Res. Commun.* **36**, 251–256

Jefcoate, C. R., Simpson, E. R., Boyd, G. S., Brownie, A. C. & Orme-Johnson, W. H. (1973) *Ann. N.Y. Acad. Sci.* **212**, 243–261

Jones, G. & DeLuca, H. F. (1975) *J. Lipid Res.* **16**, 448–453

Kodicek, E. (1974) *Lancet* **i**, 325–329

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Madhok, T. C., Schones, H. K. & DeLuca, H. F. (1978) *Biochem. J.* **175**, 479–482

Mason, H. S. (1957) *Adv. Enzymol. Relat. Areas Mol. Biol.* **19**, 79–233

Nash, T. (1953) *Biochem. J.* **55**, 416–421

Omura, T., Sato, R., Cooper, D. Y., Rosenthal, O. & Estabrook, R. W. (1965) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **24**, 1181–1189

Ritter, M. C. & Dempsey, M. E. (1971) *J. Biol. Chem.* **246**, 1536–1539

Scallen, T. J., Schuster, M. W. & Dhar, A. K. (1971) *J. Biol. Chem.* **246**, 224–230

Sladek, N. E. & Mannering, G. J. (1966) *Biochem. Biophys. Res. Commun.* **24**, 668–674

Sladek, N. E. & Mannering, G. J. (1969*a*) *Mol. Pharmacol.* **5**, 174–185

Sladek, N. E. & Mannering, G. J. (1969*b*) *Mol. Pharmacol.* **5**, 186–199

Steenbock, H. (1923) *Science* **58**, 449–450

Suda, T., DeLuca, H. F. & Tanaka, Y. (1970) *J. Nutr.* **100**, 1049–1052

Suda, T., DeLuca, H. F. & Hallick, R. B. (1971) *Anal. Biochem.* **43**, 139–146

Thomas, P. E., Lu, A. Y. H., Ryan, D., West, S. B., Kawalek, J. & Levin, W. (1976) *J. Biol. Chem.* **251**, 1385–1391