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25-Hydroxylation of Vitamin D_3 and Side Chain Hydroxylations of 5β-Cholestane-3α,7α,12α-triol by Purified Rabbit and Rat Liver Microsomal Cytochromes P-450*

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25-Hydroxylation of vitamin D_3 and side chain hydroxylations of 5β-cholestane-3α,7α,12α-triol were studied with different forms of cytochrome P-450 reconstituted with NADPH-cytochrome P-450 reductase from rabbit and rat liver microsomes.

Cytochromes P-450 LM_2 and LM_4 were prepared from liver microsomes of phenobarbital-treated rabbits. Cytochrome P-450 LM_2 showed no detectable side chain hydroxylase activities toward 5β-cholestane-3α,7α,12α-triol nor 25-hydroxylase activity toward vitamin D_3. Cytochrome P-450 LM_4 catalyzed 25-hydroxylation of both substrates more efficiently than the microsomes. The rate of 25-hydroxylation was considerably higher with 5β-cholestane-3α,7α,12α-triol than with vitamin D_3 as substrate.

Two apparently homogeneous fractions of cytochrome P-450 were purified from liver microsomes of phenobarbital-treated rats. The two fractions catalyzed 25-hydroxylation of vitamin D_3 as well as 25-hydroxylation, 26-hydroxylation, and other side chain hydroxylations (23ξ, 24α, 24β) of 5β-cholestane-3α,7α,-12α-triol. The C_{27}-steroid hydroxylase activities were lower than with microsomes, whereas the vitamin D_3 hydroxylase activity was higher.

One fraction of cytochrome P-450 (cytochrome P-450 A) was prepared from liver microsomes of untreated, phenobarbital-treated, and vitamin D-deficient rats, respectively. This fraction catalyzed 25-hydroxylation of vitamin D_3 and 5β-cholestane-3α,7α,12α-triol. 25-Hydroxylation of vitamin D_3 was about 50 times more efficient with the purified cytochrome P-450 than with microsomes. 25-Hydroxylation of the C_{27}-steroid was as efficient as with microsomes. Inhibition experiments indicated that 25-hydroxylation of vitamin D_3 and 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol were catalyzed by different species of cytochrome P-450.

Side chain hydroxylations are reactions essential for the conversion of vitamin D_3 into its active forms and for the biosynthesis of bile acids from cholesterol (1–3). Studies with rat liver have shown that the microsomal fraction catalyzes 25-hydroxylation of vitamin D_3 (4–8) as well as 25-hydroxylation and 26-hydroxylation of 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol (9). In addition to these hydroxylations, the microsomal fraction catalyzes 23ξ-, 24α-, and 24β-hydroxylations of 5β-cholestane-3α,7α,12α-triol (9). 25-Hydroxylation of vitamin D_3 and 26-hydroxylation of C_{27}-steroids are also catalyzed by the mitochondrial fraction of rat liver (5, 10–12). Both the mitochondrial and the microsomal side chain hydroxylations have been shown to be catalyzed by reconstituted systems containing partially purified cytochrome P-450 (13–16).

Recently it was shown in two separate studies (17, 18) that highly purified preparations of microsomal cytochrome P-450 and NADPH-cytochrome P-450 reductase catalyze 25-hydroxylation of vitamin D_3 and 25- and 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol. The present communication reports further studies on the 25-hydroxylation of vitamin D_3 and the side chain hydroxylations of 5β-cholestane-3α,7α,12α-triol with different forms of highly purified cytochrome P-450 from rat and rabbit liver microsomes.

EXPERIMENTAL PROCEDURES

Materials

25-Hydroxy[26,27-^{3}H]vitamin D_3, 160 Ci/mmol, was obtained from New England Nuclear. 5β[7β-^{3}H]Cholestane-3α,7α,12α-triol and 25-hydroxy[26-$^2H_3$]vitamin D_3 were synthesized as described previously (16, 19). 25-Hydroxyvitamin D_3 was a generous gift from Dr. J. Babcock (The Upjohn Co.). Vitamin D_3, sodium cholate,[1] dilauroylglycero-3-phosphorylcholine, and cofactors were obtained from Sigma. Octylamine-Sepharose 4B was prepared as described previously (20, 21). DEAE-Sepharose CL-6B, quaternary aminoethyl (QAE)-Sephadex. CM-Sephadex C-50, and polyacrylamide gradient slab gels, PAA 4/30, were obtained from Pharmacia, Sweden. Emulgen 913 was from Kao-Atlas, Tokyo, and Renex 690 from Atlas Chemical Co. Amberlite XAD-2 was obtained from Rohm & Haas and DEAE-cellulose (DE52) from Whatman. Hydroxylapatite (Bio-Gel HTP) was from Bio-Rad. Hydroxylapatite was mixed with an equal amount of CF-1 cellulose powder (Whatman) before chromatography.

Male rats of the Wistar strain, weighing about 200 g, and male rabbits of the New Zealand strain, weighing about 2 kg, were used. Phenobarbital was administered in the drinking water, 1 mg/ml, for 1 week. In one experiment, weanling rats were fed on a diet deficient in vitamin D (R 41, Astra Ewos, Södertälje, Sweden) for 8 weeks. This diet contained less than 0.03% (w/w) calcium and 2% (w/w) NaH_2PO_4.

Methods

Preparation of Rat Liver Microsomal Cytochrome P-450—Microsomes from untreated, phenobarbital-treated, and vitamin D-deficient rats were prepared and resuspended in sodium pyrophosphate buffer

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[1] The abbreviation and trivial names used are: cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; SDS, sodium dodecyl sulfate.

(16). Cytochromes P-450 A and B[2], were purified essentially according to Guengerich (17, 18, 22, 23).

Cytochrome P-450 Fraction C was prepared from liver microsomes of phenobarbital-treated rats as described by West *et al.* (24).

Preparation of Rabbit Liver Microsomal Cytochrome P-450—Liver microsomes from phenobarbital-treated rabbits were prepared as described above for rat liver microsomes. Cytochromes P-450 LM_2 and LM_4 were purified as described by Coon *et al.* (25) as modified by Hansson and Wikvall (18). It has been previously reported that the preparations of cytochromes P-450 LM_2 and LM_4 isolated as described by Hansson and Wikvall (18) have apparently the same properties as those described by Coon and associates (18, 25). Cytochrome P-450 was determined as described by Omura and Sato (26) and protein as described by Lowry *et al.* (27). SDS-Polyacrylamide gel electrophoresis was performed using Pharmacia gels, PAA 4/30 (18).

Preparation of Rat and Rabbit Liver Microsomal NADPH-Cytochrome P-450 Reductase—NADPH-cytochrome P-450 reductase, from phenobarbital-treated rats and rabbits, was prepared as described by Yasukochi and Masters (28) and had a specific activity of 55 and 50 units/mg of protein, respectively. Reductase activity was assayed according to Masters *et al.* (29) at 30 °C using 0.3 M potassium phosphate buffer. One unit corresponds to reduction of 1 μmol of cytochrome *c*/min.

Incubation Procedures and Analyses of Incubation Mixtures—Saturating concentrations of the different substrates, 50 to 150 μM, were incubated at 37 °C for 10 to 30 min with 0.1 to 1 nmol of cytochrome P-450, 1.5 to 3.0 units of NADPH-cytochrome P-450 reductase, 25 to 50 μg of dilauroylglycero-3-phosphorylcholine, and 1 to 2 μmol of NADPH in a total volume of 1 to 2 ml of 50 mM Tris-acetate buffer, pH 7.4, 100 mM Tris-Cl buffer, pH 7.4, or 150 mM potassium phosphate buffer, pH 7.4. The incubation procedure and analyses of incubation mixtures were the same as described previously (17, 18).

RESULTS

Highly purified cytochrome P-450 fractions were prepared from rat liver microsomes according to Guengerich (22, 23) (cytochromes P-450 A and B) and according to West *et al.* (24) (cytochrome P-450 Fraction C). Preparations of similar purity were also obtained from rabbit liver microsomes (cytochromes P-450 LM_2 and LM_4) by the procedure described by Coon *et al.* (25) as modified by Hansson and Wikvall (18).

All preparations of cytochrome P-450, except for cytochrome P-450 A, contained 18 to 20 nmol of cytochrome P-450/mg of protein (Table I) and were apparently homogeneous on SDS-polyacrylamide gel electrophoresis (Fig. 1).[3] Various preparations of cytochrome P-450 A contained 11 to 15 nmol of cytochrome P-450/mg of protein (Table I) and showed two protein bands on gel electrophoresis (Fig. 1). Attempts to purify this fraction further by chromatography on CM-Sephadex did not result in an increase in specific content of cytochrome P-450.

The highest yield of cytochrome P-450 was obtained in the preparation of cytochrome P-450 LM_2, cytochrome P-450 B, and cytochrome P-450 Fraction C. In accordance with previous work (22-25), these fractions could only be isolated in a highly purified state from phenobarbital-treated animals.

Table I summarizes the catalytic properties of the microsomal fractions and the different cytochrome P-450 fractions with respect to 25-hydroxylation of vitamin D_3 and side chain hydroxylations of 5β-cholestane-3α,7α,12α-triol. The results presented are from single experiments. However, cytochrome P-450 preparations were made at least twice and similar results were obtained. In the case of vitamin D deficiency only one cytochrome P-450 preparation was made.

[2] The different cytochrome P-450 fractions are named as originally described by Guengerich (22) (A and B), West *et al.* (24) (Fraction C), and Coon *et al.* (25) (LM_2 and LM_4).

[3] Cytochrome P-450 LM_2 appears in the same molecular weight region as the lower of the two protein bands of cytochrome P-450 A in SDS gel electrophoresis. Cytochrome P-450 LM_2 is not included in Fig. 1 since this fraction did not show catalytic activity toward vitamin D_3 and 5β-cholestane-3α,7α,12α-triol.

TABLE I

25-Hydroxylation of vitamin D_3 and side chain hydroxylations of 5β-cholestane-3α,7α,12α-triol by purified cytochrome P-450 fractions from rat and rabbit liver microsomes

The substrates (125 nmol) were incubated under standard conditions at 37 °C for 10 to 20 min with 0.1 to 0.5 nmol of cytochrome P-450, 1.0 to 3.0 units of NADPH-cytochrome P-450 reductase, 25 μg of dilauroylglycero-3-phosphorylcholine, and 2 μmol of NADPH in a total volume of 1 to 2 ml of 100 mM Tris-Cl buffer, pH 7.4 (vitamin D_3), 50 mM Tris-acetate buffer, or 150 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA (5β-cholestane-3α,7α,12α-triol). Cytochrome P-450 was prepared from liver microsomes of phenobarbital-treated animals unless otherwise stated.

Fraction	Cytochrome P-450		Hydroxylation of			
	Specific content	Yield	Vitamin D_3 (25)	5β-Cholestane-3α,7α,12α-triol		
				25	26	Other
	nmol/mg protein	%	*pmol/nmol cytochrome P-450 × min*			
Rat						
Microsomes	1.5	100	≤1	109	50	55
Cytochrome P-450A[a]	11.7	2.5	6	83	≤1	≤1
Cytochrome P-450A (untreated)[a]	12.1	4.0	47	151	≤1	≤1
Cytochrome P-450A (vitamin D deficient)[a]	15.3	5.0	34	130	≤1	≤1
Cytochrome P-450B[a]	18.9	6.0	4	35	7	21
Cytochrome P-450 Fraction C[b]	18.3	11.0	3	46	15	25
Rabbit						
Microsomes	2.8	100	≤1	245	≤1	≤1
Cytochrome P-450 LM_2[c]	18.5	5.5	≤1	≤1	≤1	≤1
Cytochrome P-450 LM_4[c]	19.5	3.5	3	357	≤1	≤1

[a] Prepared as described by Guengerich (22, 23).
[b] Prepared as described by West *et al.* (24).
[c] Prepared as described by Coon *et al.* (25) as modified by Hansson and Wikvall (18).

25-Hydroxylation of Vitamin D_3—Rat and rabbit liver microsomes showed very low 25-hydroxylase activities toward vitamin D_3. The maximal rate of reaction was 1 pmol of 25-hydroxyvitamin D_3 formed/nmol of cytochrome P-450 per min.

Cytochrome P-450 A was the most active in 25-hydroxylation of vitamin D_3. Preparations of cytochrome P-450 A from untreated and vitamin D-deficient rats were more active than preparations from phenobarbital-treated rats. 25-Hydroxylation of vitamin D_3 was about 50 times more efficient in the most active cytochrome P-450 fractions than in microsomes. Cytochrome P-450 B and cytochrome P-450 Fraction C catalyzed 25-hydroxylation of vitamin D_3 at lower rates than cytochrome P-450 A but more efficiently than the microsomal fraction. The lower rates of 25-hydroxylation obtained with these cytochromes P-450 might be due to a further metabolism of primarily formed 25-hydroxyvitamin D_3. However, as analyzed by thin layer chromatography, incubation of trace amounts of radioactive 25-hydroxyvitamin D_3 (10) with cytochrome P-450 A as well as cytochromes P-450 B and C resulted in the formation of 10% to 25% more polar products. No attempts were made to identify these products.

Rabbit liver cytochrome P-450 LM_2 showed no significant 25-hydroxylase activity toward vitamin D_3, whereas cytochrome P-450 LM_4 catalyzed a low but significant 25-hydroxylation.

25-Hydroxylation of 5β-cholestane-3α,7α,12α-triol—5β-Cholestane-3α,7α,12α-triol was efficiently 25-hydroxylated by rat and rabbit liver microsomes.

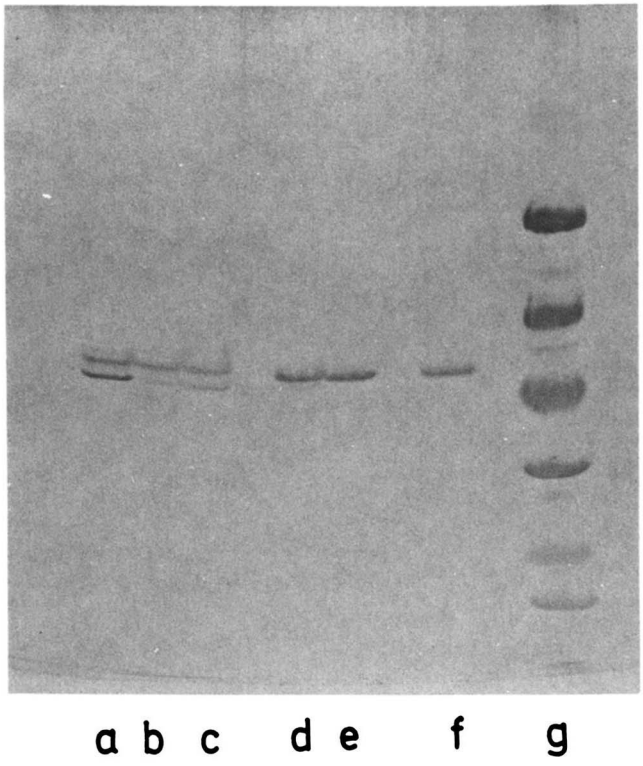

FIG. 1. **Polyacrylamide slab gel electrophoresis of purified cytochrome P-450 fractions from rat and rabbit liver microsomes.** Electrophoresis was performed in the presence of SDS on Pharmacia gradient gel PAA 4/30. The fractions were treated with sodium dodecyl sulfate and β-mercaptoethanol at 100 °C for 2 min and then subjected to electrophoresis. The amount of protein applied to the gel was 1 μg and the migration was from top to bottom. The fractions were (see Table I) (*A*) cytochrome P-450 A from phenobarbital-treated rats; (*B*) cytochrome P-450 A from untreated rats; (*C*) cytochrome P-450 A from vitamin D-deficient rats; (*D*) cytochrome P-450 B; (*E*) cytochrome P-450 Fraction C; (*F*) cytochrome P-450 LM_4; (*G*) mixture of phosphorylase *b* (M_r = 94,000), albumin (M_r = 67,000), ovalbumin (M_r = 43,000), carbonic anhydrase (M_r = 30,000), trypsin inhibitor (M_r = 20,100), and β-lactalbumin (M_r = 14,000), 3 μg of each.

Cytochrome P-450 A was the most active of the rat liver cytochrome P-450 fractions in 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol. The rate of reaction was almost the same as with the microsomal fraction. Cytochrome P-450 A prepared from untreated and vitamin D-deficient rats was slightly more active than cytochrome P-450 prepared from phenobarbital-treated rats. Cytochrome P-450 B and cytochrome P-450 Fraction C catalyzed 25-hydroxylation at lower rates than cytochrome P-450 A as well as the microsomal fraction.

Rabbit liver cytochrome P-450 LM_2 showed very low ability to catalyze 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol. Cytochrome P-450 LM_4 catalyzed 25-hydroxylation more efficiently than the microsomal fraction.

26-Hydroxylation and Other Side Chain Hydroxylations of 5β-Cholestane-3α,7α,12α-triol—Rat liver microsomes catalyzed efficient 26-hydroxylation as well as other side chain hydroxylations (23ξ,24α,24β) of 5β-cholestane-3α,7α,12α-triol. Rabbit liver microsomes showed very low ability to catalyze these hydroxylations. Cytochrome P-450 A showed no detectable 23ξ-, 24α-, 24β-, or 26-hydroxylase activities. Cytochrome P-450 B and cytochrome P-450 Fraction C catalyzed efficient hydroxylations in these positions but at slower rates than the microsomal fraction.

26-Hydroxylation or other side chain hydroxylations were not observed with cytochromes P-450 LM_2 and LM_4 from rabbit liver microsomes.

Assay Conditions for 25-Hydroxylation of Vitamin D_3—Assay conditions were worked out for 25-hydroxylation of vitamin D_3 using cytochrome P-450 A from untreated rats (Fig. 2, *A*, *C*, *E*, and *G*). 25-Hydroxylation of vitamin D_3 was approximately linear with the amount of cytochrome P-450 up to about 0.15 nmol and with time up to 15 min. The system was saturated with 3 units of NADPH-cytochrome P-450 reductase and with 10 μM concentration of substrate. At a

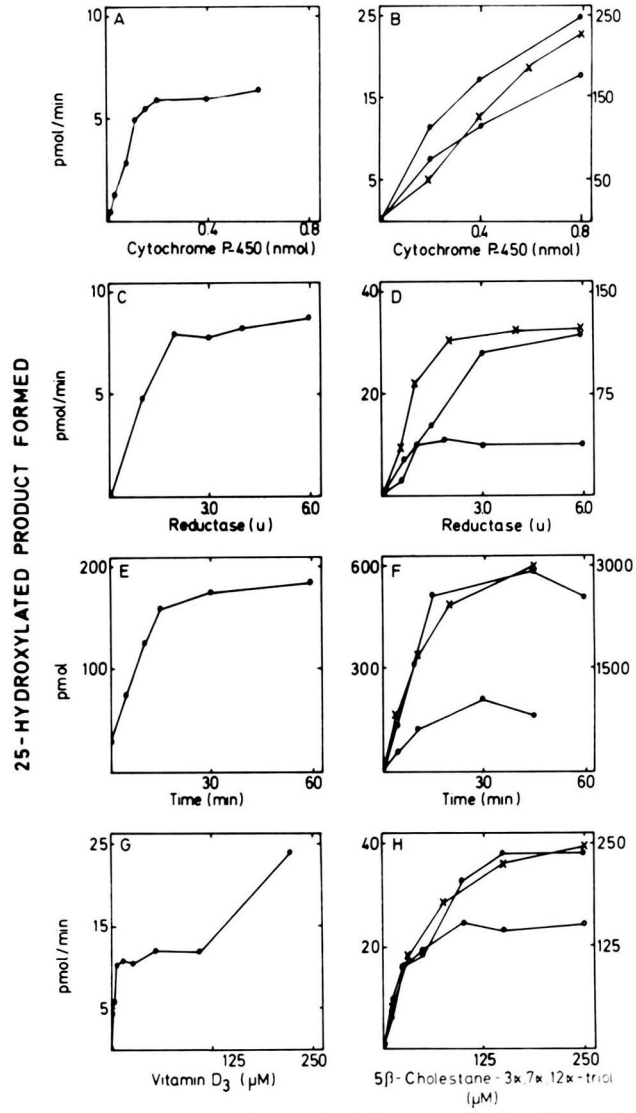

FIG. 2. **Effect of cytochrome P-450 concentration (*A* and *B*), NADPH-cytochrome P-450 reductase concentration (*C* and *D*), time (*E* and *F*), and substrate concentration (*G* and *H*) on the rate of 25-hydroxylation of vitamin D_3 (*A*, *C*, *E*, and *G*) and 5β-cholestane-3α,7α,12α-triol (*B*, *D*, *F*, and *H*).** Standard incubation procedure was used. Except when the concentration of a component was varied, the incubation mixture contained cytochrome P-450 (0.2 nmol with vitamin D_3 as substrate and 0.5 nmol with 5β-cholestane-3α,7α,12α-triol as substrate), NADPH-cytochrome P-450 reductase (2.5 units) dilauroylglycero-3-phosphorylcholine (25 μg), substrate (125 nmol), and NADPH (2 μmol) in a total volume of 1.5 ml of 50 mM Tris-acetate or 150 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. Incubations with vitamin D_3 were performed in 100 mM Tris-Cl buffer, pH 7.4. The incubation time was 10 to 20 min. The source of cytochrome P-450 A was untreated rats (25-hydroxylation of vitamin D_3) and phenobarbital-treated rats (25-hydroxylation of 5β-cholestane-3α,7α,12α-triol). ●—●, Cytochrome P-450 A; ○—○, cytochrome P-450 B; ×—×, cytochrome P-450 LM_4. The *left ordinate* applies to cytochrome P-450 A and B and the *right ordinate* applies to cytochrome P-450 LM_4.

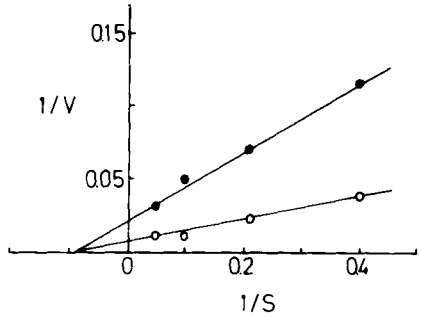

FIG. 3. **Lineweaver-Burk plots for the 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol in the absence (○——○) and in the presence (●——●) of vitamin D_3 (130 μM).** Incubation medium contained 0.5 nmol of cytochrome P-450 A from untreated rats, 2.5 units of NADPH-cytochrome P-450 reductase, 25 μg of lipid, and varying amounts of substrate. Time of incubation was 10 min and standard incubation procedure was used. *S*, concentration of 5β-cholestane-3α,7α,12α-triol (micromolar); *V*, rate of 25-hydroxylation (picomoles/nmol of cytochrome P-450 × min).

substrate concentration higher than 100 μM, the rate of hydroxylation was considerably increased compared to the plateau level. This phenomenon was observed in three independent experiments. The apparent K_M value was found to be 5 μM. Omission of phospholipid in the reconstituted system did not significantly reduce the rate of vitamin D_3 25-hydroxylation.

Assay Conditions for 25-Hydroxylation of 5β-Cholestane-3α,7α,12α-triol—Assay conditions for 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol were worked out with cytochrome P-450 A from phenobarbital-treated rats, cytochrome P-450 B, and cytochrome P-450 LM_4 (Fig. 2, *B*, *D*, *F*, and *H*). 25-Hydroxylation was linear with the amount of cytochrome P-450 up to about 0.8 nmol and with time up to about 15 min, irrespective of the form of cytochrome P-450 used. Saturating amounts of NADPH-cytochrome P-450 reductase for 25-hydroxylation catalyzed by cytochrome P-450 A, cytochrome P-450 B, and cytochrome P-450 LM_4 were 3.0, 1.5, and 3.0 units, respectively. Substrate saturation was obtained at concentrations of 150 μM to 250 μM 5β-cholestane-3α,7α,12α-triol. The apparent K_M for 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol was about 10 μM with cytochrome P-450 A, about 20 μM with cytochrome P-450 B, and about 70 μM with cytochrome P-450 LM_4. Omission of phospholipid in the reconstituted system did not reduce the rate of 25-hydroxylation significantly with any of the cytochromes P-450.

Inhibition Experiments—When an equimolar amount of vitamin D_3 was added to incubations of 5β-cholestane-3α,7α,12α-triol with cytochrome P-450 A, no inhibition of 25-hydroxylation was observed. Similarly, 25-hydroxylation of vitamin D_3 by cytochrome P-450 A was not found to be inhibited by addition of an equimolar amount of 5β-cholestane-3α,7α,12α-triol. However, addition of an excess of vitamin D_3 inhibited 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol and an excess of 5β-cholestane-3α,7α,12α-triol inhibited 25-hydroxylation of vitamin D_3.

Fig. 3 shows an experiment in which a fixed amount of vitamin D_3 (130 μM) was used as an inhibitor for 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol, catalyzed by cytochrome P-450 A from untreated rats. The inhibition was not of a competitive nature, but rather of a noncompetitive nature. In a similar experiment performed with cytochrome P-450 A from phenobarbital-treated rats, the same type of inhibition was observed.

DISCUSSION

The apparently homogeneous forms of cytochrome P-450 used in the present study contained 18 to 20 nmol of cytochrome P-450/mg of protein and have been prepared according to previously described procedures (18, 22–25). As previously reported (18), rabbit liver cytochrome P-450 LM_2 showed no detectable side chain hydroxylase activities toward 5β-cholestane-3α,7α,12α-triol nor 25-hydroxylase activity toward vitamin D_3. Cytochrome P-450 LM_4, on the other hand, catalyzed a very efficient 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol, which under optimal conditions was more efficient than with microsomes. In addition, the cytochrome P-450 LM_4 fraction catalyzed a low but detectable 25-hydroxylation of vitamin D_3. These results confirm the involvement of cytochrome P-450 LM_4 in 25-hydroxylation of C_{27}-steroids (18) and suggest a possible involvement of this fraction also in vitamin D_3 25-hydroxylation.

Cytochrome P-450 B and the cytochrome P-450 prepared as described by West *et al.* (24) (Fraction C) are known to be induced by phenobarbital treatment and are present in very low concentrations in liver microsomes of untreated rats (22–24). The catalytic activities of these cytochrome P-450 fractions have not previously been compared. They have similar apparent molecular weights on gel electrophoresis and the present results indicate that the two cytochrome P-450 fractions have similar catalytic activities in the side chain hydroxylation reactions studied. Thus, both fractions catalyzed 25-hydroxylation of vitamin D_3. Furthermore, both fractions were able to catalyze 25-hydroxylation, 26-hydroxylation, and other side chain hydroxylations of 5β-cholestane-3α,7α,12α-triol. The rates of C_{27}-steroid hydroxylations were lower with the cytochromes P-450 than with microsomes. It should be noted that the 25-hydroxylation of vitamin D_3 and 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol were catalyzed at markedly lower rates than the other hydroxylations studied. It may be pointed out that 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol (9, 15) is not stimulated by phenobarbital treatment of rats, whereas 25-hydroxylation and other side chain hydroxylations of the C_{27}-steroid are slightly stimulated by this treatment.

In addition to the apparently homogeneous cytochrome P-450 fractions, which could only be obtained from phenobarbital-treated rats, a more heterogeneous fraction, cytochrome P-450 A, was prepared. Cytochrome P-450 A was the most active of the rat liver cytochrome P-450 fractions with respect to 25-hydroxylation. No other side chain hydroxylations occurred with this fraction. Inhibition experiments indicated that 25-hydroxylation of vitamin D_3 and 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol were catalyzed by different species of cytochrome P-450. Cytochrome P-450 A was prepared from untreated, phenobarbital-treated, and vitamin D-deficient rats. Since considerable variations in vitamin D_3 25-hydroxylase activity were obtained in different preparations from groups of rats with the same type of treatment, no definitive conclusions could be drawn from these experiments. The results did not, however, support the contention that the vitamin D status of the animals is of major importance for the microsomal activity (30, 31). Thus, one of the preparations of cytochrome P-450 A from untreated rats had about the same activity as a similar preparation from vitamin D-deficient rats. The highest yield and the highest specific cytochrome P-450 content were obtained when cytochrome P-450 A was prepared from vitamin D-deficient rats. An increased amount of cytochrome P-450 in liver microsomes of vitamin D-deficient rats has been observed previously (32). It should be pointed

out that the apparent K_M for 25-hydroxylation of vitamin D_3 with cytochrome P-450 A (5 μM) is of the same magnitude as the value reported for mitochondrial 25-hydroxylation (33). However, the present K_M value is more than 10 times higher than the K_M values reported for 25-hydroxylation of vitamin D_3 in microsomes (6, 8).

A marked difference in the rates of 25-hydroxylation of vitamin D_3 and 5β-cholestane-3α,7α,12α-triol between microsomes and purified cytochrome P-450 was observed. The rate of 25-hydroxylation in the microsomal fraction was more than 100 times higher with 5β-cholestane-3α,7α,12α-triol than with vitamin D_3. The rate of C_{27}-steroid 25-hydroxylation with purified cytochrome P-450 fractions was the same or lower than with the microsomal fraction. However, the rate of vitamin D_3 25-hydroxylation was up to 50 times higher with purified cytochrome P-450 fractions than with the microsomal fraction. Similar marked enhancement of hydroxylation rate with purified cytochrome P-450 fractions has been described previously for demethylation of benzphetamine (34). Since vitamin D_3 25-hydroxylation in microsomes is very low, inhibitory factors seem to be present in the microsomal fraction. These factors which are removed in the purification procedures might possibly be of regulatory importance in the metabolism of vitamin D_3.

26-Hydroxylation of 5β-cholestane-3α,7α,12α-triol, which is efficiently catalyzed by rat liver microsomes, was considerably lower with the purified cytochrome P-450-fractions. It is possible that species of cytochrome P-450 specific for 26-hydroxylation are lost during the purification procedures. It is also possible that components in microsomes other than cytochrome P-450 and NADPH-cytochrome P-450 reductase may be required for maximal 26-hydroxylase activity.

It is evident that 25-hydroxylation of vitamin D_3 and 5β-cholestane-3α,7α,12α-triol is catalyzed by more than one species of cytochrome P-450 in rat and rabbit liver microsomes. The different cytochromes P-450 have markedly different K_M values for 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol, indicating different affinity for this substrate. It should be mentioned that overlapping substrate specificities by different forms of cytochrome P-450 in hydroxylation of substrates such as testosterone, drugs, and various xenobiotics have been previously described (22, 34–38).

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Addendum—Since the submission of this manuscript, P. S. Yoon and H. F. DeLuca have reported on the resolution and reconstitution of soluble components of rat liver microsomal vitamin D_3 25-hydroxylase ((1980) *Arch. Biochem. Biophys.* **203,** 529–541). The present results are in principle in agreement with those of Yoon and DeLuca. However, the preparations of cytochrome P-450 and NADPH-cytochrome P-450 reductase used in the present report are of a higher purity than those used by Yoon and DeLuca and the rates of 25-hydroxylation of vitamin D_3 observed are several hundred-fold higher than those observed by Yoon and DeLuca.

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