Partial purification and characterization of 5β-cholestane-3α,7α,12α-triol and 5β-cholestane-3α,7α-diol 27-monooxygenase

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Abstract Hepatic mitochondrial cytochrome P-450 has been partially purified from the non-induced rat liver. The purification consisted of solubilization with cholate, polyethylene glycol fractionation, chromatographic separation using ω-amino-*n*-octyl Sepharose 4B column, and chromatography on hydroxylapatite. The overall purification of the enzyme from the solubilized extract was about 22-fold on the basis of specific content of cytochrome P-450, and 50-fold on the basis of specific activity. The partially purified enzyme was active for both 5β-cholestane-3α,7α,12α-triol and 5β-cholestane-3α,7α-diol. That the enzyme activities for these substrates were not due to two different enzymes but to the same active site of a single enzyme protein was shown by several pieces of evidence, *i.e.*, behavior to thermal inactivation, pH-dependency of the reaction velocities, experiments with mixed substrates, and behavior towards inhibitors and activators. The lower K_m value and the higher V_{max} for 5β-cholestane-3α,7α,12α-triol compared to 5β-cholestane-3α,7α-diol seem to be important factors for the regulation mechanism that keeps the ratio of cholic acid/chenodeoxycholic acid constant in rat bile.—**Atsuta, Y., and K. Okuda.** Partial purification and characterization of 5β-cholestane-3α,7α,12α-triol and 5β-cholestane-3α,7α-diol 27-monooxygenase. *J. Lipid Res.* 1982. **23:** 345–351.

Supplementary key words cholic acid • chenodeoxycholic acid • adrenodoxin • cytochrome P-450

Both cholic acid and chenodeoxycholic acid are primary bile acids which are synthesized from cholesterol in the liver. The proposed biosynthetic pathway leading to these bile acids divides at 7α-hydroxycholest-4-en-3-one, from which the two seemingly independent pathways are derived, one leading to cholic acid and the other to chenodeoxycholic acid (1). However, the ratio of cholic acid/chenodeoxycholic acid in rat bile is kept strictly constant at 4:1 under physiological conditions (2). This may hardly be expected if the two pathways are operating independently. Instead, it seems likely that both pathways are under strict regulation. Several steps may be involved in the regulation of biosynthesis of these two bile acids, *e.g.*, at 12α-hydroxylation and at 27-hydroxylation. Of these, the latter reaction is crucially important for oxidation and cleavage of the side chain. To clarify the mechanism of regulation of 27-hydroxylation, we have studied both 5β-cholestane-3α,7α,12α-triol (THC) 27-monooxygenase (EC 1.14.13.15) and 5β-cholestane-3α,7α-diol 27-monooxygenase (DHC). In a previous paper (3), we suggested that both enzymes are similar or identical, based on the criteria described by Dixon and Webb (4). However, most of the experiments carried out to verify this hypothesis were done using mitochondria as an enzyme source. There were, therefore, some ambiguities about whether the apparent enzyme activities observed were actually dependent on enzyme activity or on permeability of substrates into the mitochondrial membrane, since the apparent enzyme activities were dependent on the rate-limiting step of the two processes. Therefore, for further confirmation of the hypothesis that the enzymes were identical or similar, it was necessary to work with a solubilized enzyme, or better yet, with the purified enzyme. In this paper we describe the partial purification of the enzyme, and provide some confirmatory evidence concerning the identity of the two enzymes in experiments carried out with the solubilized and partially purified enzyme.

EXPERIMENTAL PROCEDURES

Preparation of mitochondrial membrane fraction

Mitochondria (2.5 g of protein) were prepared from livers of non-induced male Wistar rats weighing 150–200 g, according to the method described by Wilgram and Kennedy (5). They were suspended in 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA, to give a concentration of 25 mg of protein per

Abbreviations: Cholic acid, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholan-24-oic acid; lithocholic acid, 3α-hydroxy-5β-cholan-24-oic acid; THC, 5β-cholestane-3α,7α,12α-triol; DHC, 5β-cholestane-3α,7α-diol; MHC, 5β-cholestan-3α-ol; PCMB, p-chloromercuribenzoate; EDTA, ethylenediaminetetraacetate.

ml and sonicated with cooling in an ice bath at 15-sec intervals for a total period of 3 min at 20 KHz and 60 watts output. The sonicated sample was centrifuged at 105,000 *g* for 1 hr. After the supernatant was carefully removed, the remaining pellet (0.9 g of protein) was used as the membrane fraction for further purification.

Isolation of cytochrome P-450 from non-induced rat liver mitochondria

All the manipulations were conducted at 0–4°C. Potassium phosphate buffer (100 mM, pH 7.4) containing 20% glycerol was used throughout and is referred to as 100 mM buffer etc. hereafter. The solubilization and purification procedures were carried out with some modification of the method described previously (6). The membrane fraction from non-induced rat liver mitochondria was suspended in a protein concentration of about 14 mg per ml in 100 mM buffer containing 1% sodium cholate, 1 mM EDTA, 100 mM KCl, 10 μM THC, and 0.1 mM NADPH. After standing for 1 hr, the suspension was centrifuged at 105,000 *g* for 1 hr. The resultant supernatant fraction was carefully collected and precipitated by adding 50% polyethylene glycol (dissolved in water at a concentration of 50% (w/v)). The fraction that precipitated from 0 to 25% of polyethylene glycol was collected by centrifugation at 100,000 *g* for 20 min and dialyzed overnight against 50 volumes of 50 mM buffer. Sodium cholate was added to the dialyzed polyethylene glycol fractions to a concentration of 0.5%, and was applied to an ω-amino-octyl Sepharose 4B column (2.5 × 20 cm) previously equilibrated with 100 mM buffer containing 1 mM EDTA and 0.5% sodium cholate. After extensive washing with the equilibration medium, the protein fraction containing cytochrome P-450 was eluted with 100 mM buffer containing 0.4% sodium cholate and 0.1% Emulgen 913. The combined eluates were diluted 4-fold with 20% glycerol and applied to a hydroxylapatite column (2.5 × 5 cm) equilibrated with 25 mM buffer containing 0.2% Emulgen 913. The column was washed successively with 35 mM buffer containing 0.2% Emulgen 913 and the cytochrome P-450 was then eluted with 150 mM buffer containing 0.2% Emulgen 913. The eluate was dialyzed three times against 30 volumes of 20 mM buffer. The dialyzate was then applied to a small hydroxylapatite column (0.9 × 3 cm) equilibrated with 20 mM buffer containing 0.2% Emulgen 913, and the enzyme was eluted with 200 mM buffer containing 0.2% Emulgen 913. The content of cytochrome P-450 of the best preparation obtained was 2.0 nmol/mg of protein, but in most cases it was from 0.5 to 1.2 nmol of cytochrome/mg of protein.

Analytical procedures

The reconstitution of steroid 27-monooxygenase system was performed as follows. The standard reaction mixture contained 10 pmol of the partially purified liver mitochondrial cytochrome P-450, 0.1 unit of bovine adrenodoxin reductase, 5 nmol of adrenodoxin, 30 μmol of potassium phosphate buffer (pH 7.7), 0.5 μmol of NADPH, and 20 nmol of steroid dissolved in 10 μl of acetone (except 3α-O-succinyl-5β-[24-^{14}C]cholestane-7α,12α-diol which was dissolved in 10 μl of ethanol), in a final volume of 0.5 ml. In most cases, the reaction was initiated by adding the steroid after preincubating the mixture for 5 min at 30°C, and terminated by adding 50 μl of 0.1 N HCl. When [^{3}H]THC was used as substrate, the incubation was conducted for 10 min; other substrates were incubated for 20 min. The reaction product was extracted with 2 ml of ethyl acetate. The extract was washed with water and the solvent was evaporated to dryness under N_2. The residue was dissolved in 0.2 ml of acetone and subjected to thin-layer chromatography. The solvent systems were ethyl acetate–acetone 7:3 (v/v) when cholestanetriol was used as substrate; benzene–ethyl acetate 1:2 (v/v) when 5β-[11,12-^{3}H]cholestane-3α,7α-diol was used; benzene–ethyl acetate 2:1 (v/v) when 5β-[11,12-^{3}H]cholestan-3α-ol was used; and isooctane–ethyl acetate–acetic acid 4:8:0.1 (v/v) when 3α-O-succinyl-5β-[24-^{14}C]cholestane-7α,12α-diol was used. Reference compounds used were MHC, DHC, THC, 5β-cholestane-3α,27-diol, 5β-cholestane-3α,7α,27-triol, 5β-cholestane-3α,7α,12α,27-tetrol, and 3α-O-succinyl-5β-cholestane-7α,12α-diol. The distribution of radioactivity on the thin-layer chromatograms was monitored by scanning with an Aloka radiochromatogram scanner (model TLC-1, Tokyo), and the conversion to bile acids was calculated by estimating, from a chart record, the ratio of the product peak areas to the whole. When radioactivity of products was too low to measure by a scanner, the thin-layer plate was divided into zones, and silica gel on each zone was scraped into a counting vial containing a scintillation cocktail consisting of 0.4% 2,5-diphenyloxazole and 0.01% 2,2′-*p*-phenylene-bis-(5-phenyloxazole) in toluene, and radioactivity was measured by a scintillation spectrometer (Aloka Co., Tokyo, model 903).

Steroid 27-monooxygenase activity in intact mitochondria was assayed as follows. One ml of the standard reaction mixture contained 60 nmol of steroid dissolved in 10 μl of acetone, 3.0 μmol of $MgCl_2$, 1.0 μmol of KCN, 50 μmol of potassium phosphate buffer (pH 7.0), 2.0 μmol of DL-isocitrate, and an appropriate amount of mitochondria. The reaction was started by adding steroid after preincubation of the reaction mixture for 5 min at 37°C, and terminated by adding 0.1 ml of 1 N HCl. The reaction products were extracted and estimated as described above.

Other procedures

Spectrophotometric measurements were conducted by using a spectrophotometer (JASCO Co., Tokyo, model

UVIDEC-505) equipped with a recorder. Cytochrome P-450 was estimated from the CO-difference spectrum as described by Omura and Sato (7). Protein was determined by the method of Lowry et al. (8) with bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out according to the method described by Dunker and Rueckert (9).

Materials

NADPH-adrenodoxin reductase (10) and adrenodoxin (11) were kindly supplied by Dr. S. Takemori of Hiroshima University. 5β-[^{3}H]Cholestane-3α,7α,12α-triol, 5β-[11,12-^{3}H]cholestane-3α,7α-diol, and 5β-[11,12-^{3}H]cholestan-3α-ol were synthesized according to the methods described in a previous paper (12). The specific radioactivity of 5β-[^{3}H]cholestane-3α,7α,12α-triol was 102 dpm $\times$ ng^{-1}, that of 5β-[11,12-^{3}H]cholestane-3α,7α-diol 30 dpm $\times$ ng^{-1}, and that of 5β-[11,12-^{3}H]cholestan-3α-ol 4 dpm $\times$ ng^{-1}. 3-O-Succinyl-5β-[24-^{14}C]cholestane-7α,12α-diol was synthesized by incubating 5β-[24-^{14}C]cholestane-3α,7α,12α-triol with succinic anhydride in pyridine according to the method described by Heusser and Wuthier (13), and was purified by preparative thin-layer chromatography on Kiesel gel G, using phase system isooctane–ethyl acetate–acetic acid 4:8:0.1 (v/v). The specific radioactivity was 0.16 dpm $\times$ ng^{-1}. Metapyrone and aminoglutethimide were the gift of Ciba Geigy Corp. Phenyl isocyanide was prepared according to the method described by Nef (14). Cholic acid, purchased from Sigma Chemical Co. (St Louis, MO) was twice recrystallized from 50% ethanol. Polyethylene glycol 6000 was obtained from Baker Chemical Co. (Phillipsburg, NJ). Emulgen 913 was from Kao Atlas Co. (Tokyo). Hydroxylapatite was purchased from Bio-rad Lab. (Richmond, CA). Octylamine-substituted Sepharose 4B gel was prepared according to the method described by Cuatrecasas (15). Dilauroylglyceryl-3-phosphocholine was obtained from Serdary Res. Lab. (London, Canada) and an aqueous suspension of the compound was sonicated before use.

RESULTS

Isolation of cytochrome P-450 from non-induced rat liver mitochondria

The result of partial purification of cytochrome P-450 from non-induced rat liver mitochondria is shown in **Table 1.** The method was modified from that reported in the previous paper (6). Thus, in the present experiment, the total mitochondrial membrane fraction instead of the inner membrane fraction was solubilized, because the mitochondrial outer membrane fraction from non-induced rat was found to contain little cytochrome P-450 (16). Concentration of cytochrome P-450 by precipitation with polyethylene glycol minimized the amount of cytochrome(s) P-450 which passed through the affinity column without retention. Furthermore, the addition of THC and NADPH to the solubilization medium improved the specific content of cytochrome P-450 in the eluate from the ω-amino-*n*-octyl Sepharose 4B column (data not shown). The final preparation obtained was not homogeneous judging from sodium dodecyl sulfate-acrylamide gel electrophoretogram, but the specific content of P-450 was increased 12–22-fold. Further purification was, however, hampered by the lability of the enzyme protein.

TABLE 1. Purification of non-induced rat liver mitochondrial cytochrome P-450

Preparation	Total Protein	Cytochrome P-450		
		Total Content	Specific Content	Yield
	mg	*nmol*	*nmol/mg of protein*	%
Cholate extract; polyethylene glycol fraction	478.8	43.2	0.09	100
ω-Amino-*n*-octyl Sepharose 4B column eluate	71.1	13.5	0.19	31.1
Hydroxylapatite column I eluate	23.2	11.34	0.49	26.3
Hydroxylapatite column II eluate	3.96	4.14	1.05	9.6

Effect of thermal treatment on 27-monooxygenation for THC and for DHC

To study the effect of partial denaturation due to thermal treatment of both THC and DHC 27-monooxygenase, the partially purified cytochrome P-450 preparation (oxidized form) was heated at several temperatures and then subjected to reconstitution of enzyme activity using NADPH-adrenodoxin reductase, adrenodoxin, and NADPH. As shown in **Fig. 1,** the reaction rate of THC 27-monooxygenase decreased sharply as the temperature rose and a similar curve was obtained with the activity for DHC 27-monooxygenase. Since the thermal treatment at different temperatures should have resulted in a partial denaturation at a part of enzyme protein that was affecting the enzyme activities, the present results seem to suggest that the active sites, or their juxtapositions, of these enzymes are quite similar or identical.

Effect of pH on 27-monooxygenation for THC and DHC

To study the effect of pH on both THC and DHC 27-monooxygenase, reconstitution of enzyme activities

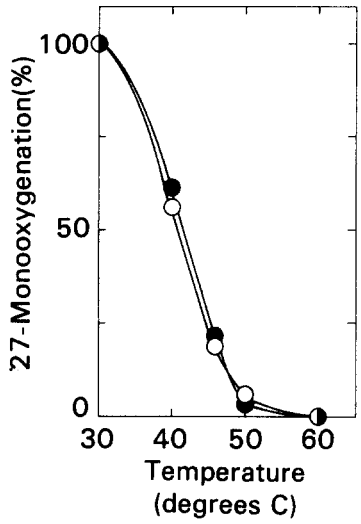

Fig. 1. Effect of thermal treatment of rat liver mitochondrial cytochrome P-450 on 27-monooxygenation of THC and DHC. The partially purified enzyme was heated at several temperatures for 5 min, and then subjected to reconstitution of 27-monooxygenation for both sterols. The reconstitution was carried out as described in Experimental Procedures. O, 27-Monooxygenation for THC; ●, 27-monooxygenation for DHC.

was carried out at different pH values. As shown in **Fig. 2,** both enzyme activities showed the same pH optimum at pH 7.7, and furthermore their profiles were quite similar to each other.

Effect of substrate concentration

The effect of substrate concentration on 27-monooxygenase activity either for THC or for DHC was studied using the reconstituted system as described under Experimental Procedures. As shown in **Fig. 3,** apparent saturation curves were observed from which the K_m values for THC and DHC were estimated to be 4.5 and 10 μM, respectively.

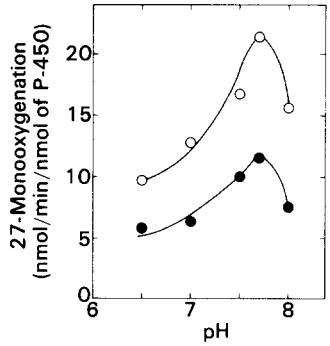

Fig. 2. Effect of pH on 27-monooxygenation of THC and DHC by rat liver mitochondrial cytochrome P-450. Incubations were conducted as described in Experimental Procedures except buffers of different pH values were used. O, 27-Monooxygenation for THC; ●, 27-monooxygenation for DHC.

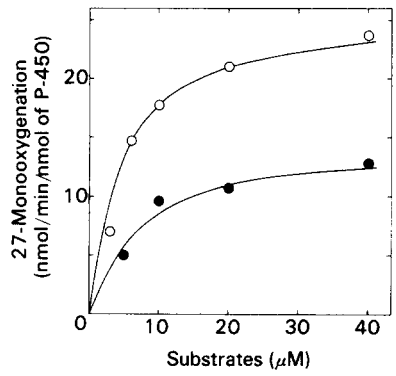

Fig. 3. Effect of substrate concentration on 27-monooxygenase activities for THC and DHC. Incubations were conducted as described in Experimental Procedures except different substrate concentrations were used. O, 27-Monooxygenation for THC; ●, 27-monooxygenation for DHC.

Mixed substrate experiments

The effect of THC on DHC 27-monooxygenase activity and vice versa was investigated by adding nonradioactive THC or DHC to the incubation mixture containing either 11,12-^{3}H-labeled DHC or ^{3}H-labeled THC, respectively, and the enzyme activity was measured for the radioactive substrate. As shown in **Fig. 4,** the presence of THC inhibited the reaction of DHC 27-monooxygenase, whereas the activity of THC 27-monooxygenase was only slightly inhibited by DHC. **Fig. 5** shows an experiment designed according to the method described by Dixon and Webb (17). As shown in the figure, it was apparently a competitive inhibition. The K_i value calculated for THC as inhibitor was 4 μM and was in good agreement with the K_m value of the sterol.

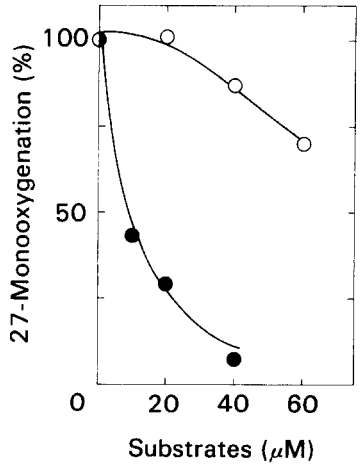

Fig. 4. Effect of THC on 27-monooxygenation of DHC and vice versa. The assay conditions were as described in Experimental Procedures, except two substrates, one radioactive (at a constant concentration of 20 μM) and the other unlabeled (at different concentrations), were used simultaneously; the reaction was started by adding NADPH. The enzyme activities were assayed only for radioactive substrates. O, 27-Monooxygenation for THC; ●, 27-monooxygenation for DHC.

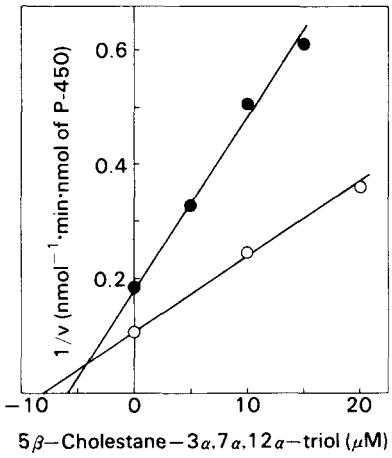

Fig. 5. Characterization of the type of inhibition of THC on 27-monooxygenase for DHC. The assay conditions were as described in the legend to Fig. 4, except the radioactive DHC was incubated at two different concentrations. ●, Experiment performed at 6 μM DHC; ○, experiment performed at 20 μM DHC.

The combined rate of 27-monooxygenation for both THC and DHC was also obtained using the two radioactive substrates, 24-^{14}C-labeled THC and ^{3}H-labeled DHC, both at nearly saturating concentrations. The enzyme activities were measured at the same time, and it was found that the total rates of reaction were not additive (data not shown).

Effect of inhibitors and calcium ion on 27-monooxygenation either for THC or for DHC

Table 2 summarizes the effects of inhibitors on 27-monooxygenase of THC and DHC. Aminoglutethimide (1 mM), which inhibits the activity of beef adrenal mitochondrial cytochrome P-450$_{scc}$ (18), showed no inhibitory effect on the two 27-monooxygenase activities; and metapyrone (1 mM), which is known to inhibit beef adrenal mitochondrial cytochrome P-450$_{11\beta}$ (19), showed only slight inhibition. On the other hand, PCMB and phenyl isocyanide inhibited the reaction completely at a concentration of 1 mM. In the reconstituted system, calcium ion inhibited both 27-monooxygenase activities to some extent at concentrations much lower than those which cause the dissociation of adrenodoxin and adrenodoxin reductase (**Table 3**) (20).

Substrate specificity

As shown in **Table 4,** 27-monooxygenase activity decreased in the order of THC, DHC, 3α-O-succinyl-5β-cholestane-7α,12α-diol, and MHC. These results clearly demonstrate that THC is the most favorable substrate of the compounds tested, DHC is the second, and MHC is the least favorable. All three hydroxyl groups at C_3, C_7, and C_{12} seem to be necessary for the high turnover number of the enzyme for THC, of which the one at C_7 may be most important because loss of hydroxyl group at C_7 resulted in marked loss of activity (about 75%). The hydroxyl group at C_3 also seems to be important because blocking it with succinate resulted in significant loss of activity.

Effect of detergent on 27-monooxygenase either for THC or for DHC

Effects of some detergents on 27-monooxygenase at different concentrations are shown in **Fig. 6.** Detergents such as Emulgen, cholate, deoxycholate, and Triton N-101 showed a similar effect on 27-monooxygenase both for THC and DHC; deoxycholate revealed the most intensive inhibitory effect. Emulgen showed the mildest inhibition compared to the others and cholate showed a moderate inhibition.

DISCUSSION

Owing to very low content of cytochrome P-450 and high content of other types of hemoprotein in rat liver mitochondria, the purification of cytochrome P-450 from this source was a considerably difficult task, and the preparation obtained was still not homogeneous as judged by electrophoresis. However, the overall purification from a soluble extract was improved over that described in a previous report (6) by applying the several

TABLE 2. Effect of inhibitors on 27-monooxygenases

Inhibitors	Concentration	Inhibition of 27-Monooxygenase for 5β-Cholestane-3α,7α,12α-triol	Inhibition of 27-Monooxygenase for 5β-Cholestane-3α,7α-diol
	mM	%	%
Metyrapone	1.0	19	25
Aminoglutethimide	1.0	0	9
p-Chloromercuribenzoate	1.0	100	100
Phenyl isocyanide	1.0	100	100

The assay conditions were as described in Experimental Procedures, except 0.5 μmol of inhibitors were added to the incubation mixture.

TABLE 3. Effect of calcium ion on 27-monooxygenases

$CaCl_2$	27-Monooxygenase Activity for	
	5β-Cholestane-3α,7α,12α-triol	5β-Cholestane-3α,7α-diol
μM	%	%
None	100	100
10	79	87
100	12	21
1000	0	0

The assay conditions were as described in Experimental Procedures, except incubations were conducted in the presence of different concentrations of calcium ion.

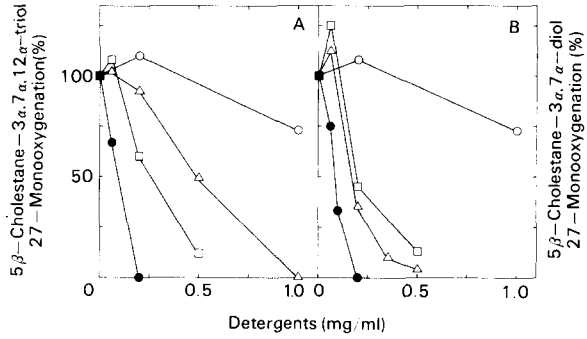

Fig. 6. Effect of detergent on 27-monooxygenation of THC and DHC. Incubations were conducted as described in Experimental Procedures, except detergents were added to the incubation mixture at different concentrations. Since the partially purified cytochrome P-450 contained Emulgen at a concentration that gave rise to a final concentration of 0.01 mg/ml, the effect of detergents described is an additional one. ○, Emulgen 913; △, sodium cholate; □, Triton N-101; ●, sodium deoxycholate.

modifications described in the previous section. Thus the best preparation of cytochrome P-450 obtained in the present experiment contained 2.0 nmol of protoheme/mg of protein, and the overall purification from the soluble extract was about 22-fold based on the specific content of cytochrome P-450 and about 50-fold based on the specific activity. These values are higher than those attained with the highly purified preparation of liver microsomal cytochrome P-450 (6–8-fold based on the specific content of cytochrome P-450). Recently, a preparation of rat liver mitochondrial cytochrome P-450, capable of both vitamin D_3 25-hydroxylation and THC 27-monooxygenation, was reported by Björkhem and Holmberg (21). However, we could not elicit any conclusion from their data as to whether their preparation was the same enzyme as ours or not. Their preparation was different in the specific content of cytochrome P-450 (1:13) and in the specific activity (1:25), and we had not measured vitamin D_3 25-hydroxylation activity in our preparation.

In the solubilization of cytochrome P-450 in rat liver mitochondria, the ratio of cholate to protein was crucially important, and the best results were obtained at a cholate/protein ratio of 0.5–0.8/1, w/w. An increase of this ratio resulted in solubilization of other types of hemoprotein that hampered the further purification, while a decrease resulted in incomplete solubilization of cytochrome P-450. Other kinds of affinity columns such as Sepharose 4B with cholesterol as ligand or with THC as ligand were also tried. Although in some cases these affinity columns gave better results than the amino-*n*-octyl Sepharose column, they were not superior in most cases. Ion exchange chromatography was also tried, but no additional purification was achieved. Further purification of the partially purified enzyme was hampered by the instability of the enzyme.

The partially purified enzyme showed 27-monooxygenation activity for THC, DHC, and MHC, respectively, when it was incubated with adrenodoxin, NADPH-adrenodoxin reductase, and NADPH. The hypothesis raised in the previous report, that THC and DHC 27-monooxygenation are catalyzed by a single enzyme and therefore are the same enzyme, is now highly substantiated by the following observations obtained with the partially purified enzyme. *1*) The ratios of both enzyme activities were the same in mitochondria and in the reconstituted system (Table 4). *2*) By thermal inactivation, both enzyme activities declined in a parallel fashion. *3*) The reaction velocity-pH curves of both enzyme activities were similar. *4*) When both THC and DHC were incubated together, the former behaved as com-

TABLE 4. Substrate specificity of 27-monooxygenase in the reconstituted system and in intact mitochondria

Substrate	27-Monooxygenase Activity of		
	Reconstituted System		Mitochondria
	nmol/min/nmol of cytochrome P-450	*nmol/10 min/mg of protein*	*nmol/10 min/mg of protein*
5β-Cholestane-3α,7α,12α-triol	23.2	252.2	5.4
5β-Cholestane-3α,7α-diol	11.0	119.6	2.4
5β-Cholestan-3α-ol	2.6	28.3	0.6
3α-O-Succinyl-5β-cholestane-7α,12α-diol	6.7	72.8	—

The incubations were conducted as described in Experimental Procedures.

petitive inhibitor for the latter and the K_i value of the former coincided with its K_m value and vice versa. *5*) When the above experiment was carried out using both radioactive substrates at nearly saturating concentrations, the total rates of reaction were not additive. *6*) Both enzyme activities were affected by inhibitors and activators in a similar fashion. Since the present experiment was carried out with the solubilized, partially purified enzyme, it is clear that the enzymatic activities observed were dependent on the real enzyme activities, but not on the apparent activity caused by the permeabilities of substrates into the mitochondrial membrane.

In normal rat bile, the ratio of cholic acid/chenodeoxycholic acid is kept constant at 4:1. Although the mechanism of this regulation is so far unknown, there seem to be some regulatory points in the biosynthetic pathways of these bile acids. One may be at the hydroxylation at C_{12} and another at C_{27}-mono-oxygenation. Since in the present experiment it was shown that THC 27-monooxygenase is the same enzyme as DHC 27-monooxygenase, the regulation exerted at this point seems to be kinetic, and is dependent on the values of K_m, V_{max}, and substrate concentration for the enzyme reactions. The fact that the 27-monooxygenase for THC has lower K_m value and higher V_{max} than that for DHC (Table 4) seems to suggest that these kinetic values are the important factors for the control of the ratio of cholic acid/chenodeoxycholic acid in rat bile.

It was recently established in this laboratory that the reconstituted system from rat liver mitochondrial cytochrome P-450 hydroxylates the methyl group of THC in position 27 (25-pro-S methyl group) (22) and therefore the name of the enzyme was used as 27-monooxygenase instead of 26-monooxygenase which has been used up to this time.

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