Formation of chenodeoxycholic acid from 3α,7α-dihydroxy-5β-cholestanoic acid by rat liver peroxisomes

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Abstract The oxidation of the side chain of 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA) into chenodeoxycholic acid has been studied in subcellular fractions of rat liver. The product was separated from the substrate by high pressure liquid chromatography and identified by gas–liquid chromatography–mass spectrometry. The highest specific rate of conversion was found in the heavy (M) and the light (L) mitochondrial fractions with the highest enrichment in the L fraction. Washing the M fraction reduced the side chain cleavage activity by 90%. The peroxisomal marker enzyme urate oxidase was reduced to the same extent. The activity found in the M fraction may thus be due to peroxisomal contamination. After centrifugation of the L fraction on a Nycodenz density gradient, the highest specific activity for side chain cleavage of DHCA (31 nmol × mg^{-1} × h^{-1}) was found in the fraction with the highest peroxisomal marker enzyme activity. This fraction also catalyzed conversion of 3α,7α,12α-5β-cholestanoic acid (THCA) into cholic acid at the highest rate (32 nmol × mg^{-1} × h^{-1}). The peroxisomal oxidation of DHCA into chenodeoxycholic acid required the presence of ATP, CoA, Mg^{2+}, and NAD in the incubation medium. The reaction was not inhibited by KCN. It is concluded that rat liver peroxisomes contain enzymes able to catalyze the cleavage of the side chain of both DHCA and THCA. The enzymes involved are similar to, but not necessarily identical to, those involved in the peroxisomal β-oxidation of fatty acids. —**Prydz, K., B. F. Kase, I. Björkhem, and J. I. Pedersen.** Formation of chenodeoxycholic acid from 3α,7α-dihydroxy-5β-cholestanoic acid by rat liver peroxisomes. *J. Lipid Res.* 1986. **27:** 622–628.

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According to current concepts, the formation of cholic acid and chenodeoxycholic acid from cholesterol involves intermediate formation of 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) and 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA), respectively (1–3). Recently we found that formation of cholic acid from THCA was catalyzed most efficiently by the peroxisomal fraction of rat liver homogenate (4, 5) with intermediate formation of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid (6). This conversion was shown to have a cofactor requirement similar to that for the peroxisomal β-oxidation of fatty acids (5). It has been assumed that the mechanism of side chain oxidation of DHCA is the same as that of THCA (1–3). Gustafsson (7) reported that rat liver microsomes in the presence of the 100,000 *g* supernatant and ATP converted DHCA into 3α,7α,24-trihydroxy-5β-cholestanoic acid. This compound was efficiently converted into chenodeoxycholic acid in the bile fistula rat (7). He concluded that the mechanism for conversion of DHCA to chenodeoxycholic acid appeared to be the same as that of THCA to cholic acid (7). In vivo studies by Cass, Williams, and Hanson (8) showed that THCA had the ability to competitively inhibit side chain oxidation of DHCA in the hamster, indicating that DHCA and THCA share at least one enzyme in the side chain oxidation.

Whether or not the cleavage of the side chain of THCA and DHCA is catalyzed by the same or by separate enzyme systems can not be evaluated from the above studies. So far, no in vitro studies on the overall conversion of DHCA into chenodeoxycholic acid have been reported. We report here on the subcellular distribution and the cofactor requirements of the enzymes converting DHCA into chenodeoxycholic acid in rat liver. It is shown that the peroxisomal fraction is most important for the side chain cleavage of both DHCA and THCA.

MATERIALS AND METHODS

Chemicals

[7β-^{3}H]3α,7α,12α-Trihydroxy-5β-cholestanoic acid (THCA, 200 Ci/mol) and [7β-^{3}H]3α,7α-dihydroxy-5β-cholestanoic acid (DHCA, 200 Ci/mol) as well as the

Abbreviations: THCA, 3α,7α,12α-trihydroxy-5β-cholestanoic acid; DHCA, 3α,7α-dihydroxy-5β-cholestanoic acid; HPLC, high pressure liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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corresponding unlabeled compounds were prepared as previously described (7, 9, 10). All labeled compounds were purified before use by high pressure liquid chromatography (HPLC, cf. below). The R- and the S- isomers of THCA and DHCA were not separated since it was found that both isomers were equally well converted into the corresponding primary bile acids. [^{14}C]Cholic acid and [^{14}C]chenodeoxycholic acid used as standards were from the Radiochemical Centre, Amersham, England. Nycodenz and Maxidenz were from Nyegaard & Co. A/S, Oslo, Norway. Other chemicals were from Sigma Chemical Co., St. Louis, MO or E. Merck, Darmstadt, F.R.G. All solvents were of analytical or HPLC grade.

Preparation of liver subcellular fractions

Male Wistar rats (115–250 g) given a commercial pellet diet and water ad libitum were used. The liver was finely minced and homogenized in ice-cold 0.25 M sucrose, 1 mM EDTA, pH 6.5, with Trizma base (6.5 vol, v/w) by one stroke in a Potter-Elvehjem homogenizer with a loose-fitting pestle. The homogenate was centrifuged for 10 min at 2,200 rpm (600 g_{av}) in the HB 4 rotor in a Sorvall RC2-B centrifuge. The pellet was rehomogenized (3.5 vol, v/w) and the suspension was recentrifuged. The combined postnuclear supernatants were centrifuged at 6,500 rpm (4,900 g_{av}) for 10 min in the same rotor, giving the heavy mitochondrial (M) fraction as a pellet. The 4,900 g_{av} supernatant was centrifuged at 16,200 rpm (24,000 g_{av}) for 10 min in the SS-34 rotor. The resulting pellet (light mitochondrial (L) fraction) was washed once. The combined 24,000 g_{av} supernatants were centrifuged at 29,000 rpm (60,000 g_{av}) for 1 hr in the Ti 60 rotor of a Sorvall ultracentrifuge to obtain the microsomal (P) pellet. The supernatant was used as the cytosolic (S) fraction. All pellets were resuspended in 0.25 M sucrose, 15 mM HEPES, pH 7.4.

Preparation of peroxisomes

Peroxisomal fractions were prepared on a linear Nycodenz gradient from 10% (w/v) in 0.25 M sucrose, 1 mM HEPES, pH 7.4, and 1 mM EDTA to 48% in 1 mM HEPES and 1 mM EDTA in a vertical rotor (Beckman VTi 50). The light mitochondrial fraction (approximately 50 mg of protein) was layered on top of the gradient and centrifuged at 20,000 rpm (35,000 g_{av}) for 75 min in a Sorvall OTD 55B centrifuge. Fractions of 2.5 ml were collected.

The following marker enzymes were used: cytochrome *c* oxidase (11), monoamine oxidase (12), rotenone-insensitive NADPH-cytochrome *c* reductase (13), urate oxidase (14), catalase (15, 16), D-amino acid oxidase (16), β-acetylglucosaminidase (17), and glucose-6-phosphatase (18). Protein was determined by the method of Lowry et al. (19) or the modified method of Bensadoun and Weinstein (20) in fractions containing Nycodenz.

Incubation, extraction, and chromatographic procedures

Under standard conditions the incubation mixture contained the following in 0.75 ml of 0.1 M Tris-HCl buffer, pH 8.0: 2 mM CoA, 7.5 mM ATP, 10 mM $MgCl_2$, 7.5 μM FAD, and 6.7 μM [^{3}H]THCA or [^{3}H]DHCA (100,000–200,000 cpm) added in less than 5 μl of ethanol. With the main subcellular fractions, the amount of protein was usually 200 μg per incubation. With the density gradient fractions, an equal volume of 50 or 100 μl from each fraction, containing up to 700 μg of protein, was incubated. After 15 min of preincubation at 37°C, the reaction was started by the addition of 2 mM NAD^+ and the incubations were continued for 30 min. The reactions were terminated, hydrolyzed, and extracted as described (5).

Aliquots of the incubation extracts were analyzed by HPLC using a Zorbax ODS column (5.0 × 250 mm) and assayed for radioactivity as previously described (5). To locate the products in the chromatograms, 200 cpm of either [^{14}C]chenodeoxycholic acid or [^{14}C]cholic acid was used as internal standard. The extracts of the incubations with DHCA were eluted in 17% and those with THCA in 23% 30 mM trifluoroacetic acid (adjusted to pH 2.9 with triethylamine) in methanol. In these systems the elution volume of R-DHCA was 52–54 ml, that for S-DHCA was 59–60 ml, and for chenodeoxycholic acid it was 22–23 ml. The elution volume for R-THCA was 50–51 ml, 54–55 ml for S-THCA, and 20–21 ml for cholic acid.

Combined gas-liquid chromatography–mass spectrometry (GLC-MS)

Aliquots of the HPLC fractions corresponding to cholic acid and chenodeoxycholic acid were converted into methyl ester–trimethylsilyl ether derivatives and analyzed by combined GLC-MS using the same equipment and conditions as described (21, 22). The identities of cholic acid and chenodeoxycholic acid were established by monitoring the same selected ions as in previous work (22).

RESULTS

Subcellular distribution of chenodeoxycholic acid formation from 3α,7α-dihydroxy-5β-cholestanoic acid

When labeled DHCA was incubated with the main subcellular fractions, two major radioactive products appeared in the chromatograms of the extracts of incubations with both the mitochondrial and the light mitochondrial fractions (**Fig. 1A**). The elution volume of the most polar peak (22 ml) corresponded to that of chenodeoxycholic acid. The identity was confirmed by GLC-MS by monitoring the ions at *m/e* 255 and *m/e* 370. The product with retention volume 41 ml (Fig. 1A) could be reduced by $NaBH_4$ to DHCA and was most probably a result of 3α-hydroxysteroid dehydrogenase activity. The identities

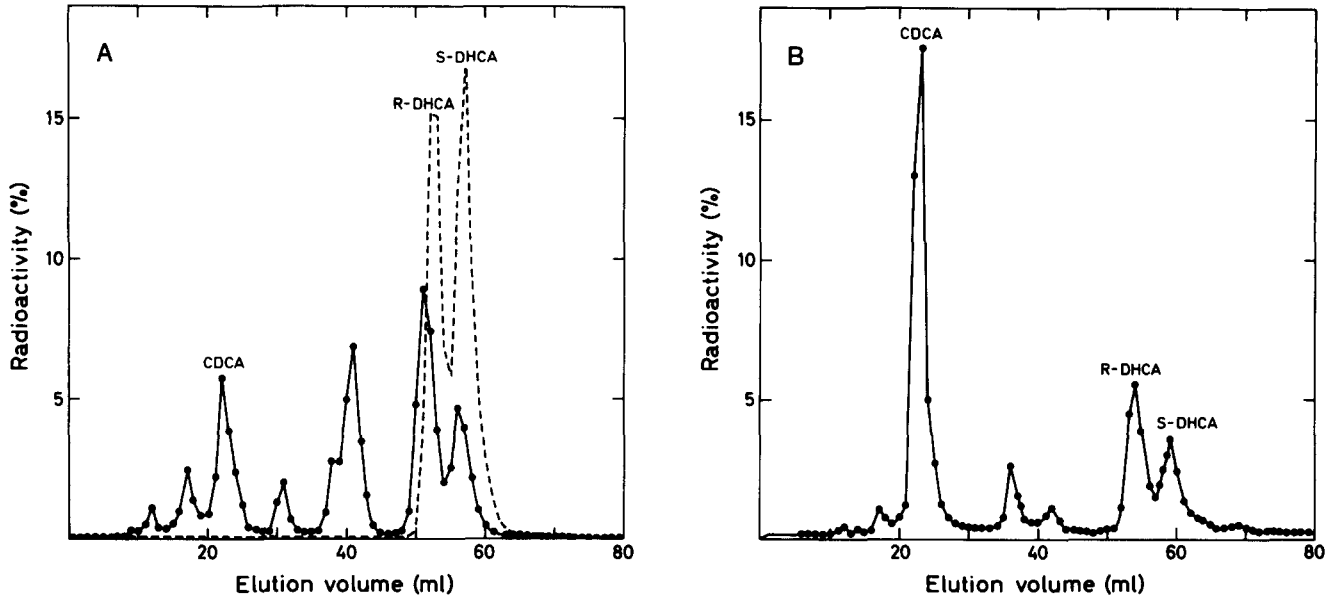

Fig. 1. Reversed phase high pressure liquid chromatogram of an extract of incubation with DHCA: A, the light mitochondrial fraction (——) and a blank incubation (- - -) and B, the peroxisomal fraction of rat liver. The incubation conditions, extraction and chromatographic procedures are given in Materials and Methods.

of other minor products were not established. The subcellular distribution of chenodeoxycholic acid formation from DHCA, as well as that of cholic acid formation from THCA, is shown in **Table 1**. The heavy mitochondrial fraction showed the highest specific activity of chenodeoxycholic acid formation, while the highest relative specific activity (% activity/% protein) was observed in the light mitochondrial fraction. The distribution of cholic acid formation from THCA (Table 1) followed entirely the distribution of the peroxisomal marker enzymes (**Table 2**), as previously shown (5). Both the highest specific activity and the highest relative specific activity were found in the light mitochondrial fraction (Table 2). In order to minimize rupture of the fragile peroxisomes, a gentle homogenization procedure was used, and the M-fraction was not washed during preparation. This may explain the high degree of peroxisomal contamination in the crude nuclear (N) fraction and the heavy mitochondrial (M) fraction (Table 2). In an experiment where the M-fraction was washed three times in the resuspension medium, the specific activity of chenodeoxycholic acid formation from DHCA and that of cholic acid from THCA was reduced to 1/10 of the activity in the unwashed fraction (**Table 3**). A corresponding reduction in the activity of the peroxisomal marker enzyme urate oxidase supports the assumption that peroxisomal contamination of the M-fraction mainly contributed to the activity of chenodeoxycholic acid and cholic acid formation.

TABLE 1. Formation of cholic acid from 3α,7α,12α-trihydroxy-5β-cholestanoic acid and of chenodeoxycholic acid from 3α,7α-dihydroxy-5β-cholestanoic acid in subcellular fractions of rat liver

	Protein		Formation of Cholic Acid				Formation of Chenodeoxycholic Acid			
Fraction	mg	%	nmol/hr	%	nmol/hr × mg	% Activity/% Protein[a]	nmol/hr	%	nmol/hr × mg	% Activity/% Protein[a]
E + N	1221	100	2599	100		1	11188	100		1
N	350	29	483	19	1.4	0.7	3637	33	10.4	1.1
M	268	22	917	35	3.4	1.6	4023	36	15.0	1.0
L	86	7	372	14	4.3	2.1	982	9	11.4	1.3
P	104	8	74	3	0.7	0.4	156	1.4	1.6	0.2
S	333	27	0	0	0	0	50	0.4	0.2	0.02
Recovery		93		71				80		

The data represent the results of one of three sets of experiments with essentially the same distribution of protein and chenodeoxycholic acid formation. E, postnuclear supernatant; N, crude nuclear fraction; M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, cytosolic fraction.

[a] % Activity/% Protein, relative specific activity.

TABLE 2. Subcellular distribution and relative specific activity[a] (in parentheses) of marker enzymes in rat liver

Fraction	Protein	Urate Oxidase		Catalase		D-Amino Acid Oxidase		Monoamine Oxidase		Cytochrome *c* Oxidase		Rotenone-insensitive NADPH-Cytochrome *c* Reductase	
	%	%		%		%		%		%		%	
E + N	100	100	(1.0)	100	(1.0)	100	(1.0)	100	(1.0)	100	(1.0)	100	(1.0)
N	29	18	(0.6)	16	(0.6)	28	(0.9)	25	(0.9)	22	(0.8)	27	(0.9)
M	22	35	(1.6)	43	(2.0)	46	(2.1)	50	(2.3)	43	(2.0)	9	(0.4)
L	7	25	(3.6)	17	(2.4)	38	(5.5)	9	(1.3)	9	(1.3)	15	(2.1)
P	8	0		0		3	(0.4)	6	(0.8)	1	(0.1)	32	(4.0)
S	27	0		29	(1.1)	10	(0.4)	0		0		1	(0.4)
Recovery	93	78		105		125		90		75		84	

The enzyme activities were measured as referred to in Materials and Methods. Fractions abbreviated as in Table 1.
[a]Relative specific activity = % activity/% protein.

When ^{3}H-labeled DHCA was incubated with a peroxisome-rich fraction from the Nycodenz density gradient, essentially one product corresponding to chenodeoxycholic acid was observed in the chromatogram (Fig. 1B). A small peak that eluted at 35 ml might represent an intermediate in the reaction, but no attempts were made to identify the material in this peak. The distribution of activity through the gradient is shown in **Fig. 2** together with the profiles of the marker enzymes, the gradient density profile, and the protein distribution. It is seen that both DHCA and THCA were most efficiently converted into chenodeoxycholic acid and cholic acid, respectively, with fractions containing the highest peroxisomal marker enzyme activity. Some differences in the distribution of chenodeoxycholic acid and cholic acid-forming activity were noted, however. The bottom, and most peroxisome-rich fraction, converted DHCA into chenodeoxycholic acid at a rate of 31 nmol · mg^{-1} · h^{-1} and accounted for 17% of the gradient's total chenodeoxycholic acid-forming activity. The same fraction converted THCA to cholic acid at a similar rate (32 nmol · mg^{-1} · h^{-1}) but accounted for 55% of the gradient's total activity. The activity of the peroxisomal marker enzyme D-amino acid oxidase in this fraction represented 29% of the total activity in the gradient. The total conversion of DHCA into chenodeoxycholic acid in all the fractions of the gradient was 560 nmol/hr compared to 170 nmol/hr for the conversion of THCA into cholic acid, corresponding to a recovery of 80% and 70%, respectively. No significant enrichment, measured as increased relative specific activity (% activity/% protein), was observed in any of the fractions containing microsomal or mitochondrial marker enzymes (fractions no. 7–15) either for cholic acid or for chenodeoxycholic acid formation.

Gradient fractions corresponding to the highest activity of peroxisomal marker enzymes were incubated with both R-DHCA and S-DHCA. The conversion to chenodeoxycholic acid was catalyzed equally well in both cases.

Cofactor requirement

We have earlier shown that conversion of THCA to cholic acid by rat liver peroxisomes has the same cofactor requirement as peroxisomal β-oxidation of fatty acids (4, 5). The results shown in **Table 4** indicate that the conversion of DHCA to chenodeoxycholic acid by purified peroxisomes is dependent on CoA, ATP, NAD, and Mg^{2+} as was found for formation of cholic acid from THCA. The reaction was not stimulated by the addition of FAD (cf. Discussion).

Factors influencing the formation of the primary bile acids

The addition of 1 mM KCN to the incubation medium did not inhibit the formation of cholic acid from THCA or chenodeoxycholic acid from DHCA. In some experiments there was even a slight stimulation of these conversions, both in the M fraction and the L fraction.

The gradient fractions with the highest activity of the peroxisomal marker enzymes (fraction 1) and the mitochondrial marker enzymes (fraction 9) were incubated with THCA and DHCA in the presence and absence of bovine serum albumin (BSA). The addition of BSA (2.7 mg/ml) did not stimulate the formation of either cholic acid or chenodeoxycholic acid in the peroxisomal fraction. In the latter case there was an apparent stimulation of formation of a minor, not identified, product more polar than chenodeoxycholic acid (elution volume 17 ml, Fig. 1B).

DISCUSSION

DHCA and THCA are intermediates in the formation of chenodeoxycholic acid and cholic acid, respectively (1–3). The oxidative cleavage of these intermediates is thought to involve a reaction sequence similar to the β-oxidation of fatty acids (1–5). It is not known whether

TABLE 3. Effect of washing the heavy (M) and the light (L) mitochondrial fractions on the formation of chenodeoxycholic acid and cholic acid from 3α,7α-dihydroxy-5β-cholestanoic acid and 3α,7α,12α-trihydroxy-5β-cholestanoic acid, respectively

Fraction	Formation of Chenodeoxycholic Acid	Formation of Cholic Acid	Urate Oxidase
	nmol/hr × *mg*	*nmol/hr* × *mg*	*units/min* × *mg*
M, unwashed	3.0	2.9	1.4
M, washed three times	0.3	0.3	0.2
L, washed once	5.5	5.6	2.8
L, washed twice	3.3	2.3	2.7

DHCA and THCA are oxidized by the same or different enzyme systems.

In previous work we have shown that the conversion of THCA into cholic acid is most efficiently catalyzed by the peroxisomal fraction of rat liver (4, 5), indicating an important role of peroxisomes in the formation of bile acids. We have also shown that in the Zellweger syndrome, a fatal inherited disease where liver peroxisomes cannot be detected, DHCA and THCA accumulate as a consequence of defective conversion into chenodeoxycholic acid and cholic acid (23, 24). This strongly indicates that liver peroxisomes are important in the normal formation of bile acids in man also.

The overall conversion of THCA and DHCA into the primary bile acids depends on a series of reactions that presumably requires an intact organelle structure. Peroxisomes are known to be fragile organelles and, in order to preserve the structure as intact as possible, the choice of isolation procedure may be critical. In previous studies we have used both Percoll and sucrose as gradient media (4, 5). The use of Nycodenz in the present work has several advantages. Nycodenz gradients are nearly iso-osmotic, and the organelles will not be subjected to an osmotic shock during isolation, as in sucrose gradients. The contamination of the peroxisome-rich fractions by microsomal protein is less in the Nycodenz gradient, and both mitochondria and lysosomes have smaller equilibrium densities in Nycodenz gradients than in sucrose gradients, while for peroxisomes the equilibrium density remains the same (25, 26).

In the present work we have shown that the formation of chenodeoxycholic acid from DHCA is also most efficiently catalyzed by the peroxisomal fraction of rat liver. The conversion of DHCA into chenodeoxycholic acid by the peroxisomal fractions from the Nycodenz gradient can hardly be explained by contamination of other organelles. Based on the ratio of the specific NADPH-cytochrome *c* reductase activity in the purified peroxisomal fractions to that in the microsomal fraction, it was estimated that a maximum of 10% of the protein in the peroxisomal fraction may be contaminating microsomal protein. A similar estimation based on glucose-6-phosphatase yielded a contamination of less than 1%. This contamination is much smaller than reported for Percoll and sucrose gradient centrifugation (4, 5). From the results shown in Table 1, it is also seen that the specific activity in the microsomal fraction is several-fold lower than that in the L fraction, which makes it highly unlikely that the activity observed is due to microsomal contamination. In a previously published report on the conversion of DHCA to 24-OH-DHCA by the microsomal fraction in combination with the 100,000 *g* supernatant, the possibility of peroxisomal contamination was not discussed (7). Lysosomal marker enzyme activity (β-AGA, 17) was not detectable in the peroxisomal fractions from the Nycodenz gradient, excluding the possibility that the side chain cleavage activity is due to lysosomal enzymes. To what extent a small DHCA side chain cleavage activity may be localized to the mitochondrial fraction is difficult to assess. A small activity towards both THCA and DHCA was observed in the fractions from the Nycodenz gradient corresponding to those with the highest mitochondrial marker enzyme activity (Fig. 2). These activities were not inhibited by KCN, which makes it unlikely that they are related to the mitochondrial β-oxidation system. The observation that there was no significant increase in relative specific activity in these fractions makes it most likely that these activities are due to a small amount of peroxisomal contamination.

The requirements in vitro for the peroxisome-catalyzed conversion of DHCA to chenodeoxycholic acid (Table 4) were the same as those previously reported for the conversion of THCA to cholic acid. The only difference was the lack of stimulation by FAD in the present work. This lack of stimulatory effect of FAD, and also of BSA, and the much higher rates of conversion obtained, may be explained by the Nycodenz gradient procedure used that, as discussed above, appears to preserve the structure of the peroxisomes better during preparation. The in vitro requirements are consistent with a reaction sequence similar to that of the peroxisomal β-oxidation of fatty acids (27). The enzymes involved should thus be an ATP-dependent 5β-cholestanoyl-CoA synthetase, an FAD-containing oxidase, a hydratase, an NAD-dependent 24-hydroxy-5β-cholestanoyl-CoA dehydrogenase, and a thiolase. In this reaction sequence intermediate formation of a Δ^{24}-unsaturated steroid followed by formation of 3α,7α,24-trihydroxy-5β-cholestanoic acid would be expected. Evi-

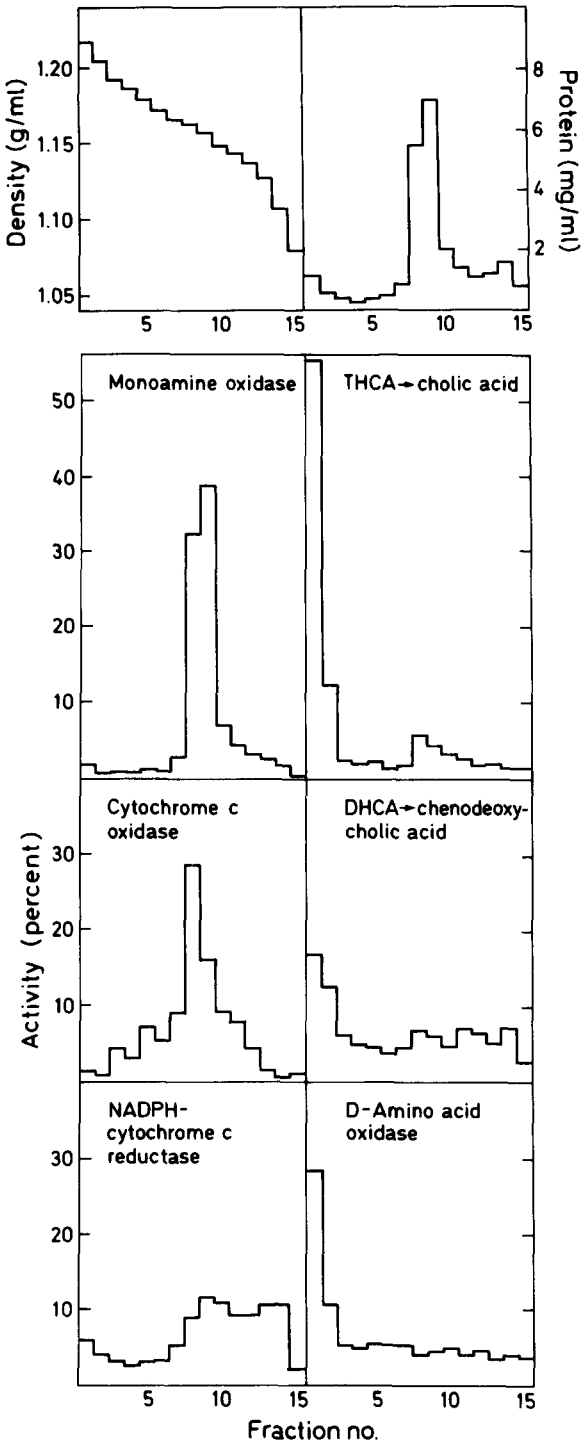

Fig. 2. Density profile and protein distribution (upper panel) and enzyme activities (lower panel) in fractions after centrifugation of the light mitochondrial fraction of rat liver on a Nycodenz density gradient. The experimental procedures are described in Materials and Methods. The gradient was fractionated into 2.5-ml fractions. Enzyme activities are expressed as percentage of the amount in the whole gradient. In gradient fraction no. 1, the specific activity for formation of cholic acid was 32 nmol • mg^{-1} • hr^{-1} and for formation of chenodeoxycholic acid was 31 nmol • mg^{-1} • hr^{-1}.

TABLE 4. Cofactor requirement for the conversion of 3α,7α-dihydroxy-5β-cholestanoic acid into chenodeoxycholic acid by rat liver peroxisomal fraction

Alterations	Formation of Chenodeoxycholic Acid
	nmol/hr × mg protein
Complete system[a]	34.9
Minus NAD	6.3
Minus ATP	1.0
Minus CoA	1.1
Minus $MgCl_2$	1.0
Minus FAD	35.8
Minus all cofactors	0.9

[a]The peroxisomal fraction corresponding to 20 μg of protein was incubated with 4.7 nmol of DHCA as described in Materials and Methods.

dence has been obtained for the formation of such intermediates in the peroxisomal formation of cholic acid from THCA (6). In the present work no attempts were made to isolate possible intermediates in the reaction (peak at 35 ml, Fig. 1B). Although the reaction mechanism for the oxidation of DHCA to chenodeoxycholic acid appears to be the same as for the oxidation of THCA, we cannot, on the basis of the present experiments, decide whether the same or different peroxisomal enzyme systems are involved. More detailed kinetic studies are required to answer this question.

In summary, we have shown that DHCA and THCA are oxidized to chenodeoxycholic and cholic acid, respectively, by peroxisomal enzymes in rat liver. These findings clearly indicate that liver peroxisomes are important in the formation of the primary bile acids.

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