Human hepatoblastoma cells (HepG2) and rat hepatoma cells are defective in important enzyme activities in the oxidation of the C_{27} steroid side chain in bile acid formation

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Abstract We have examined the ability of HepG2 human hepatoblastoma cells and 7800 C1 Morris rat hepatoma cells to convert 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) and 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA) to cholic acid and chenodeoxycholic acid, respectively. Cell extracts from both these cell lines could neither form cholic acid from THCA nor from the activated form, THCA-CoA. This suggests that both cell lines are defective in two enzyme activities involved in the pathway, the microsomal THCA-CoA ligase and the peroxisomal THCA-CoA oxidase. Furthermore, we show that the subsequent enzymes are active in the conversion to bile acids, because the product of the THCA-CoA oxidase, 3α,7α,12α-trihydroxy-5β-cholest-24-enoyl-coenzyme A (Δ24-THCA-CoA) or Δ24-THCA in the presence of THCA-CoA ligase, are converted to cholic acid by both cell lines. HepG2 cells were able to slowly form chenodeoxycholic acid and cholic acid from 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol, respectively, in 24- and 96-h incubations. The rate of cholic acid formation was lower than the rate for chenodeoxycholic acid and there was a clear accumulation of THCA. 7800 C1 Morris cells had no ability to form cholic acid or chenodeoxycholic acid after 96 h incubation. We conclude that these two cell lines have defects in two enzyme activities involved in the peroxisomal oxidation in bile acid formation, the microsomal THCA-CoA ligase and the peroxisomal THCA-CoA oxidase.—**Östlund Farrants, A-K., A. Nilsson, and J. I. Pedersen.** Human hepatoblastoma cells (HepG2) and rat hepatoma cells are defective in important enzyme activities in the oxidation of the C_{27} steroid side chain in bile acid formation. *J. Lipid Res.* 1993. **34:** 2041–2050.

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Cell cultures of both hepatocytes and hepatoma cell lines are widely used in the study of bile acid synthesis and regulation (1–6). Hepatoma cells, such as human hepatoblastoma HepG2 cells (5) and HUH6-C1-5 cells (6), in particular, have been used by several workers to perform long-term studies, thus avoiding the problem of the limited life span of hepatocytes. In most of the studies using HepG2 cells, a different bile acid pattern was found than that found in human liver. Cholic acid and chenodeoxycholic acid, the main primary bile acids synthesized in humans, are synthesized in approximately equal amounts in human liver (7). In most studies with HepG2 cells, chenodeoxycholic acid is the predominant primary bile acid formed, with a lower amount of cholic acid secreted into the medium (8–10). In addition to these two primary bile acids, a C_{27} bile acid precursor for cholic acid, 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA), has been found secreted into the medium. In other reports, however, cholic acid and chenodeoxycholic acid have been synthesized in equal amounts (11, 12).

The small amount of cholic acid formed by HepG2 cells and the presence of THCA in the medium have led to the suggestion that these cells have a defect late in bile acid synthesis (8). The CoA ester of THCA is the substrate for the last series of reactions in the formation of primary bile acids, the oxidation of the C_{27} steroid side chain (for review, see ref. 13). These reactions have been found to occur in the peroxisomes (14–16) by a reaction mechanism similar to that of the peroxisomal β-oxidation of fatty acids (**Fig. 1**) (17, 18).

In this study we have examined the ability of HepG2 cells to form cholic acid and chenodeoxycholic acid. Of

Abbreviations: THCA, 3α,7α,12-trihydroxy-5β-cholestanoic acid; DHCA, 3α,7α-dihydroxy-5β-cholestanoic acid; Δ24-THCA, 3α,7α,12α-trihydroxy-5β-cholest-24-enoic acid; 24-OH-THCA, 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid; LDCF, 2'7'-dichlorofluorescine diacetate; PMSF, phenylmethyl sulfonyl fluoride; DTT, dithiothreitol; TFA, trifluoroacetic acid; HPLC, high performance liquid chromatography; GC–MS, gas chromatography–mass spectrometry.

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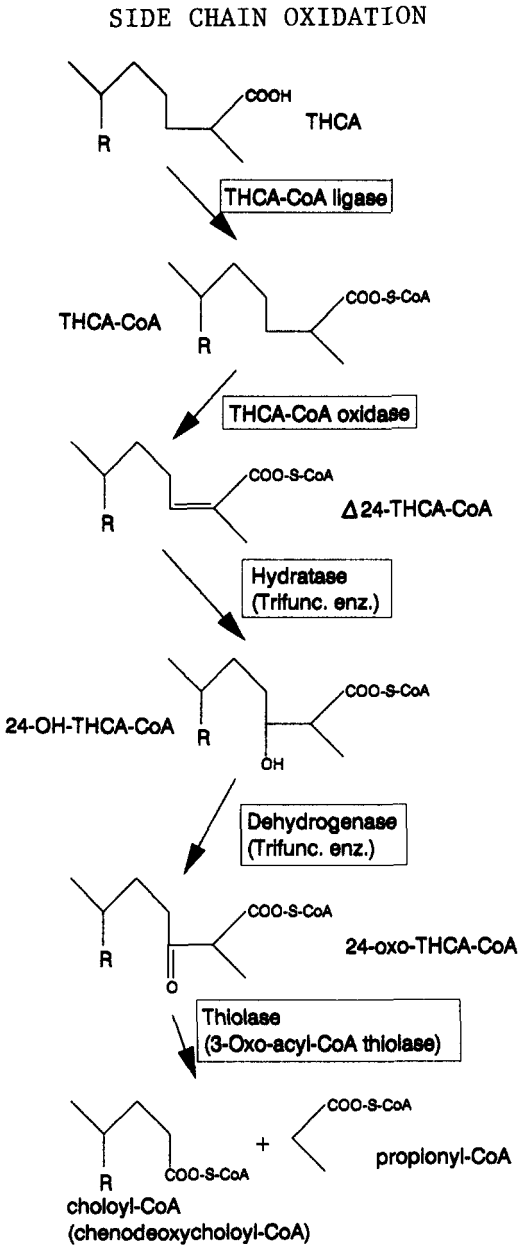

Fig. 1. Proposed reaction mechanism for the side chain cleavage in the conversion of 5β-cholestanoic acid into primary bile acids. 3α,7α,12α-Trihydroxy-5β-cholestanoic acid, THCA (and also 3α,7α-dihydroxy-5β-cholestanoic acid, DHCA) is first activated to the CoA-ester by the microsomal THCA-CoA ligase. The subsequent steps occur in the peroxisome. The bile acids produced are cholic acid from THCA or chenodeoxycholic acid from DHCA. The proposed enzymes involved are in boxes.

particular interest has been the ability of these cells to activate and oxidize THCA and 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA) to cholic acid and chenodeoxycholic acid, respectively. HepG2 cells have several of the normal peroxisomal activities found in hepatocytes, including the peroxisomal β-oxidation (19, 20). In addition to HepG2 cells, 7800 C1 Morris rat hepatoma cells have also been investigated for these peroxisomal activities. These cells have a peroxisomal β-oxidation, which is susceptible to induction by peroxisome proliferators (21, 22). The results presented here show that HepG2 cells have a detectable cholesterol 7α-hydroxylase activity, the initial and rate-limiting step in bile acid synthesis (for review, see ref. 13), although it is low compared to rat hepatocytes. However, HepG2 cells are defective in important enzyme activities for the oxidation of THCA and DHCA. The same defects are found in 7800 C1 Morris cells.

MATERIALS AND METHODS

Chemicals

3α,7α,12α-Trihydroxy-5β-[7β-^{3}H]cholestanoic acid, 3α,7α-dihydroxy-5β-[7β-^{3}H]cholestanoic acid and the unlabeled compounds were prepared from alligator bile as previously described (23). Purification of the substrates was performed by HPLC on a Zorbax ODS column (0.46 × 25 cm) using as eluting solvent 17% 30 mM trifluoroacetic acid (TFA) (pH 2.9, with triethylamine) in methanol. Synthetic 3α,7α,12α-trihydroxy-5β-cholest-24-enoic acid (Δ24-THCA) was prepared by treatment of 3α,7α,12α-triacetoxy-5β-cholan-24-al with α-carbetoxyethyl-phosphorane as described (24), and purified by HPLC on a 5-μm Nucleosil C-18 column (25 × 0.4 cm) using 24% 30 mM TFA in methanol. Δ24-THCA-CoA was formed biologically by incubating Δ24-THCA with an enriched THCA-CoA ligase preparation which is described below. The CoA ester was isolated and purified by HPLC on a 10-μm Nucleosil C-18 column (15 × 0.4 cm) using 34% isopropanol in 10 mM sodium acetate buffer at pH 6.0. Palmitoyl-CoA, clofibric acid, dexamethasone (cell culturing grade), retinoic acid, and retinol were obtained from Sigma, MO, and 2',7'-dichlorofluorescine diacetate (LDCF) was from Eastman Kodak, NY. Culture media, fetal calf serum, and horse serum were from Flow Laboratories, Irvin, Scotland. All solvents were of analytical grade. Other chemicals were commercial high purity material.

Hepatocyte preparation and culture conditions

Livers from male Wistar rats (200 g) were perfused as described by Blomhoff and Berg (25). The hepatocytes were plated out on 60 mm petri dishes in Dulbecco's modification of Eagle's medium (DMEM) containing 2% Ultroser G, gentamicin (0.1 mg/ml) and L-glutamine (2 mM).

HepG2 cells, purchased from American Type Culture Collection, Rockville, MD were cultivated in DMEM containing 10% fetal calf serum, gentamicin (0.1 mg/ml) and L-glutamine (2 mM). 7800 C1 Morris rat hepatoma cells were cultivated in 1 × Ham's F-10 medium containing 10% horse serum, 2% fetal calf serum, gentamicin (0.1 mg/ml) and L-glutamine (2 mM). Both cell lines were trypsinized 3 times weekly.

All cells were incubated at 37°C in a humidified air–CO_2 95:5 (v/v) atmosphere and harvested in 50 mM potassium phosphate buffer at pH 7.5, frozen, and sonicated 2 × 5 sec prior to use.

Incubation of HepG2 cells and 7800 C1 Morris cells

HepG2 cells and 7800 C1 Morris cells were maintained in the media described above in 250-ml and 100-ml flasks, respectively, until they were confluent or near confluent. The media for HepG2 and 7800 C1 Morris cells were then replaced by 10 ml and 4 ml, respectively, of serum-free media containing a mixture of amino acids and either 50 μg (11.3 μM) [^{3}H]THCA or 20 μg (4.4 μM) [^{3}H]DHCA. The cells were incubated for 96 h. The media and the cells were hydrolyzed separately in alkali overnight, extracted, and analyzed as described under analytical procedures. HepG2 cells were also incubated with 50 μg 5β-cholestane-3α,7α,12α-triol or 50 μg 5β-cholestane-3α, 7α-diol for 24 h or 96 h in the medium described above. The media and cells were combined before hydrolysis and extraction.

Preparation of THCA-CoA ligase

A solubilized preparation of THCA-CoA ligase was prepared from the microsomal fraction as described by Schepers et al. (26) with modifications as described by Östlund Farrants, Andersen, and Byström (27). The microsomal fraction was prepared as described in 0.25 M sucrose, 2 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride (PMSF), and 1 mM dithiothreitol (DTT). The microsomal fraction was washed once in solubilization buffer (1 mM DTT, 1 mM EDTA, 4 mM $MgCl_2$, 10 mM MOPS at pH 7.2) containing 1 M NaCl and centrifuged at 120,000 g_{av} for 40 min. The microsomes were then treated with solubilization buffer containing 1% Triton X-100 followed by solubilization buffer containing 1 M NaCl and 1% Triton X-100. The microsomes were left on ice for 30 min during both treatments and centrifuged as above after treatment. The supernatant from the last treatment is referred to as the enriched THCA-CoA ligase preparation.

Enzyme assays and analytical procedures

Acyl-CoA (palmitoyl-CoA) oxidase activity was assayed by determination of H_2O_2 production, coupled to the oxidation of LDCF, as described by Small, Burdett, and Connock (28) and modified by Leighton (personal communication). The oxidation of LDCF by H_2O_2 to 2',7'-dichlorofluorescein was followed spectrophotometrically at 502 nm. The reaction mixture contained 0.1 M Tris-HCl (pH 8.5), 0.05 mM LDCF, 50 μg horseradish peroxidase type II (EC 1.11.1.7), 0.015 mM FAD, 0.6 mg/ml bovine serum albumin (BSA), 0.02% Triton X-100, and was started with 60 μM palmitoyl-CoA (all final concentrations). The reaction mixture contained 20–60 μg of protein in a total volume of 1 ml at 25°C. The LDCF was prepared daily at 5.1 mM in 0.01 M NaOH and stored in a light-tight container under N_2 gas.

The conversion of C_{27} bile acid intermediates to primary bile acids was measured as described by Östlund Farrants, Björkhem, and Pedersen (29). The standard incubation mixture contained 0.1 M Tris-HCl (pH 8.0), 0.1 mM CoA, 2 mM DTT, 8.5 mM ATP, 10 mM $MgCl_2$, 75 μM FAD, 2 mg/ml BSA, 0.01% Triton X-100 and 40 μM [^{3}H]THCA, [^{3}H]Δ24-THCA or [^{3}H]DHCA (35,000 cpm) (final concentrations). NAD was added after 15 min of incubation to a final concentration of 2 mM. The incubations contained 200–600 μg of protein in a total volume of 0.25 ml. The reaction mixture was incubated at 37°C for 1 h or 90 min. The incubations were stopped with 5 μl 6 M KOH, and hydrolyzed at 60°C for 30 min to remove bound CoA. After acidification and extraction with ethyl acetate, the samples were analyzed by reversed phase HPLC on a 5 μm C-18 Nucleosil column (0.5 × 25 cm). The eluting solvent was 24% 30 mM TFA (pH 2.9 with triethylamine) in methanol for THCA and 20% 30 mM TFA in methanol for DHCA; 0.8-ml fractions were collected and assayed for radioactivity. Rates of conversion were calculated from the percentage distribution of radioactivity.

Cholesterol 7α-hydroxylase activity was measured by the HPLC-spectrophotometric method described by Ogishima and Okuda (30) and modified by Hylemon et al. (31). Rat microsomes were prepared in an isolation buffer containing 0.1 M potassium phosphate at pH 7.2, 0.1 M sucrose, 50 mM potassium chloride, 50 mM sodium fluoride, 5 mM EGTA, 3 mM DTT, 1 mM EDTA, and 1 mM PMSF as described by Hylemon et al. (31). Rat hepatocytes in suspension and HepG2 cells were sonicated in the isolation buffer described above for 2 × 10 sec. The extracts were centrifuged at 660 g_{av} to remove nuclei and cell debris. The supernatant from this centrifugation was centrifuged at 105,000 g_{av} for 2 h. The resulting pellet was resuspended in the isolation buffer and measured for cholesterol 7α-hydroxylase activity immediately. The incubation mixture contained 0.1 M potassium phosphate (pH 7.4), 50 mM sodium fluoride, 5 mM DTT, 1 mM EDTA, 20% glycerol, and 0.015% CHAPS. One or 3 mg of protein from the different fractions was added. The reaction mixture was preincubated at 37°C for 5 min before an NADPH-regenerating system was added containing sodium citrate (5 mM), $MgCl_2$ (5 mM), NADPH (0.5 mM), and isocitrate dehydrogenase (0.075 units) giving a final reaction volume of 1 ml. The reaction mixtures were incubated at 37°C for 40 min and stopped by adding 30 μl of 20% cholate. The second reaction converting the formed 7α-hydroxycholesterol to 7α-hydroxy-4-cholesten-3-one was started by adding 44 μl of 1% freshly prepared cholesterol oxidase in 10 mM potassium phosphate (pH

7.4), 1 mM DTT, and 20% glycerol. This reaction mixture was incubated for 10 min and stopped by adding 2 ml of ethyl acetate. Before extraction with ethyl acetate, 15,000 cpm (0.12 μg) of [^{14}C]cholesterol was added as an internal recovery standard. After being dried down under N_2, the samples were analyzed by HPLC on a 5-μm Nuclesil C-18 column (25 × 0.4 cm) using 20% 30 mM TFA in methanol, and 24% 30 mM TFA in methanol. The amount of product formed was determined by monitoring the absorbance at 240 nm and calculating the number of nmoles using a calibration curve.

Protein determination

Protein concentration of cell extracts was made according to Lowry et al. (32).

RESULTS

Ability of cell extracts from rat hepatocytes, HepG2 cells, and 7800 C1 Morris cells to oxidize THCA to cholic acid

The oxidation rate of THCA to cholic acid in a rat hepatocyte suspension was lower than that found in the homogenate (postnuclear fraction E) from rat liver. The oxidation rate in rat hepatocyte primary cultures 24 h after plating was only slightly lower than in hepatocyte suspension. After 4 days of culturing, the oxidation rate had dropped considerably, however, and only 30% of the activity remained (**Table 1**). The activity of acyl-CoA oxidase did not decline as dramatically as the oxidation rate of THCA during this time period (Table 1).

Both HepG2 and 7800 C1 Morris cells had detectable activities of acyl-CoA oxidase, 0.7 and 2.8 nmol min^{-1} mg^{-1}, respectively. However, no detectable amounts of cholic acid could be found when cell extracts from HepG2 cells or 7800 C1 Morris cells were incubated with THCA (**Table 2**). Addition of an enriched THCA-CoA ligase preparation did not restore the conversion of THCA to cholic acid in either cell line. Nor did any of the other intermediates of the oxidation pathway appear, such as the unsaturated form of THCA, Δ24-THCA, or 24-OH-THCA (see Fig. 1). The product of the first peroxisomal enzyme in the pathway, the Δ24-THCA, was also used as substrate, but no cholic acid was formed in incubations from either cell line. Only in the presence of the enriched THCA-CoA ligase preparation was Δ24-THCA converted to cholic acid (Table 2). Cholic acid was also formed from Δ24-THCA-CoA (Table 2). In addition, 24-OH-THCA was formed in the incubation with Δ24-THCA and the enriched preparation of THCA-CoA ligase. These findings suggest that these two cell lines are defective in two enzyme activities involved in the formation of cholic acid, the microsomal THCA-CoA ligase (26, 33), and the peroxisomal THCA-CoA oxidase (34, 35).

The 24-OH-THCA and cholic acid were formed in different relative amounts by cell extracts from the two cell lines when incubated with Δ24-THCA and THCA-CoA ligase. HepG2 gave more 24-OH-THCA than cholic acid, whereas 7800 C1 Morris cells gave more cholic acid than 24-OH-THCA (Table 2). With Δ24-THCA-CoA both cell lines showed a similar pattern with only cholic acid formed, but relatively more in 7800 C1 Morris cells.

TABLE 1. The conversion rate of THCA to cholic acid and the acyl-CoA oxidase activity

	CA Formation	Acyl-CoA Oxidase
	nmol h^{-1} mg^{-1}	*nmol min^{-1} mg^{-1}*
E-fraction (n = 4)	8.4 [0.6]	10.7 [4.1]
Suspension (n = 2)	2.2–3.4	1.54–1.65
24 h (n = 16)	2.4 [1.0]	3.7 [0.9]
4 days (n = 11)	0.74 [0.19]	2.3 [1.0]

The oxidation rate of THCA, measured as formation of cholic acid (CA), and the peroxisomal β-oxidation, measured as acyl-CoA oxidase activity, were determined in rat postnuclear E-fraction, rat hepatocytes in suspension, rat hepatocyte primary cultures 24 h and 96 h after plating. The values are means of the number of observations stated (n), and standard deviation is given in brackets.

Ability of cell extracts from HepG2 cells and 7800 C1 Morris cells to oxidize DHCA to chenodeoxycholic acid

Cell extracts of HepG2 and 7800 C1 Morris cells did not form chenodeoxycholic acid from DHCA, either on their own or together with the enriched preparation of THCA-CoA ligase. The THCA-CoA ligase preparation used was able to activate DHCA to the same extent as THCA (240 nmol h^{-1} mg^{-1} and 300 nmol h^{-1} mg^{-1}, respectively). Furthermore, the intermediate of DHCA, which is supposed to correspond to Δ24-THCA, was also given as substrate to cell extracts from these two cell lines. This intermediate was produced by incubating the S-form of DHCA with gradient purified peroxisomes in the absence of NAD and isolating it on HPLC, using a 5-μm Nucleosil column with 20% 30 mM TFA at pH 2.9 in methanol as eluting solvent (**Fig. 2**). When this intermediate was incubated with gradient-purified rat liver peroxisomes in the presence of NAD, chenodeoxycholic acid was formed. Of the two cell lines, only 7800 C1 Morris cells were able to convert this substrate into substantial amounts of chenodeoxycholic acid and then only when the THCA-CoA ligase preparation was present in the incubation mixture (1.5 nmol h^{-1} mg^{-1}). HepG2 cells converted only a very small fraction of the substrate to chenodeoxycholic acid in the presence of THCA-CoA ligase.

TABLE 2. The conversion rate of THCA and Δ24-THCA with and without the addition of an enriched THCA-CoA ligase preparation, and of Δ24-THCA-CoA

	7800 C1 Morris Cells		HepG2 Cells	
	CA	24-OH-THCA	CA	24-OH-THCA
	nmol h⁻¹ mg⁻¹		*nmol h⁻¹ mg⁻¹*	
THCA	n.d.	n.d.	n.d.	n.d.
THCA + THCA-CoA ligase	n.d.	n.d.	n.d.	n.d.
Δ24-THCA	n.d.	n.d.	n.d.	n.d.
Δ24-THCA + THCA-CoA ligase	1.46 [0.29]	0.82 [0.40]	0.36 [0.20]	0.60 [0.19]
Δ24-THCA-CoA	4.16–4.18	n.d.	0.39 [0.17]	n.d.

Cholic acid (CA) and 24-OH-THCA formation by cell extracts from 7800 C1 Morris rat hepatoma cells (n = 6) and HepG2 human hepatoblastoma cells (n = 3). The values represent means and standard deviation is given in brackets; n.d., not detectable.

Incubation of HepG2 cells in culture with 5β-cholestane-3α,7α,12α-triol, 5β-cholestane-3α,7α-diol, THCA, and DHCA

HepG2 cells were able to form cholic acid from 5β-cholestane-3α,7α,12α-triol and THCA, and chenodeoxycholic acid from 5β-cholestane-3α,7α-diol and DHCA. The HPLC patterns from extractions of both the medium and the cells from HepG2 cells incubated with THCA for 96 h showed a peak corresponding to cholic acid. However, no Δ24-THCA was found in the ethyl acetate extract from the cells (**Fig. 3a**), or in the extract from the medium (not shown). Cholic acid was also formed during incubation of the cells with 5β-cholestane-3α,7α,12α-triol for 24 h and 96 h, both rates 150 ng h^{-1}. There was a considerable accumulation of THCA after both 24 h and 96 h. After 24 h, 6.5-times more THCA than cholic acid was found secreted into the medium corresponding to a rate of 980 ng h^{-1} (Fig. 3b). The identity of the peaks was confirmed by combined gas chromatography–mass spectrometry (GC–MS).

Extracts from the combined medium and cells from HepG2 incubated with DHCA showed a more complex pattern on HPLC, with one peak corresponding to chenodeoxycholic acid (**Fig. 4a**). In addition, two large less polar peaks appeared, one with the same retention time as THCA and one eluting between chenodeoxycholic acid and THCA. Ethyl acetate extraction of the combined medium and cells from incubations with 5β-cholestane-3α,7α-diol gave a clearer pattern, i.e., DHCA, THCA, and chenodeoxycholic acid (Fig. 4b). Chenodeoxycholic acid was formed at a rate of 270 ng h^{-1}, but no DHCA could be detected. Both 5β-cholestane-3α,7α,12α-triol and THCA were found in this extract; THCA was formed at a rate of 120 ng h^{-1}, and 5β-cholestane-3α,7α,12α-triol at 100 ng h^{-1} at 24 h. No cholic acid could be detected in this extract.

Incubation of 7800 Morris cells in culture with THCA, and DHCA and treatment with THCA, clofibric acid, retinoids, dexamethasone or fatty acids in the culture medium

No bile acids or intermediates of either THCA or DHCA were found in media from incubations with 7800 C1 Morris cells (not shown). In an attempt to restore the

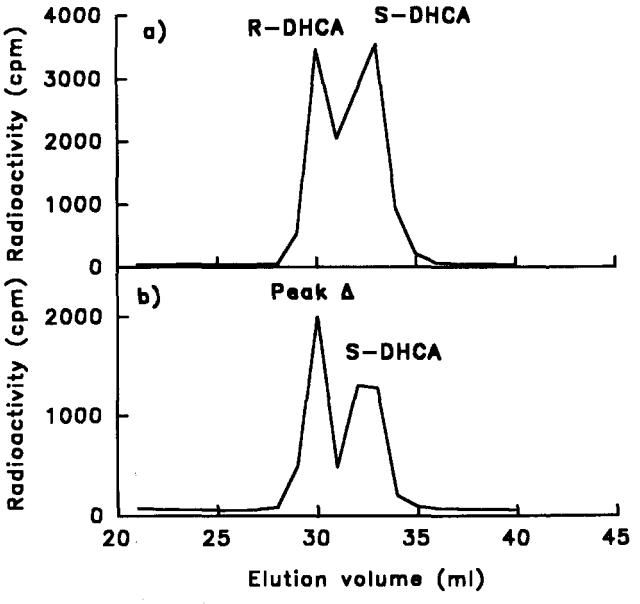

Fig. 2. HPLC profiles of a) DHCA and b) an incubation of gradient-purified peroxisomes with S-DHCA in the absence of NAD. Fifty μg (0.9 10^6 cpm) of [^{3}H]DHCA was injected onto the column in several portions a), and after extraction with ethyl acetate 10 μg (180,000 cpm) of S-THCA was incubated with 60 μg of gradient-purified peroxisomes in the absence of NAD as described for the conversion of C_{27} bile acid intermediates under Materials and Methods. The peak marked "Peak Δ" was then purified and isolated using HPLC. The column was a 5 μm Nucleosil C-18 and the solvents were 20% 30 mM TFA in methanol. One μg of peak Δ was retrieved after purification, and was used in incubations with cell extracts as described under Material and Methods.

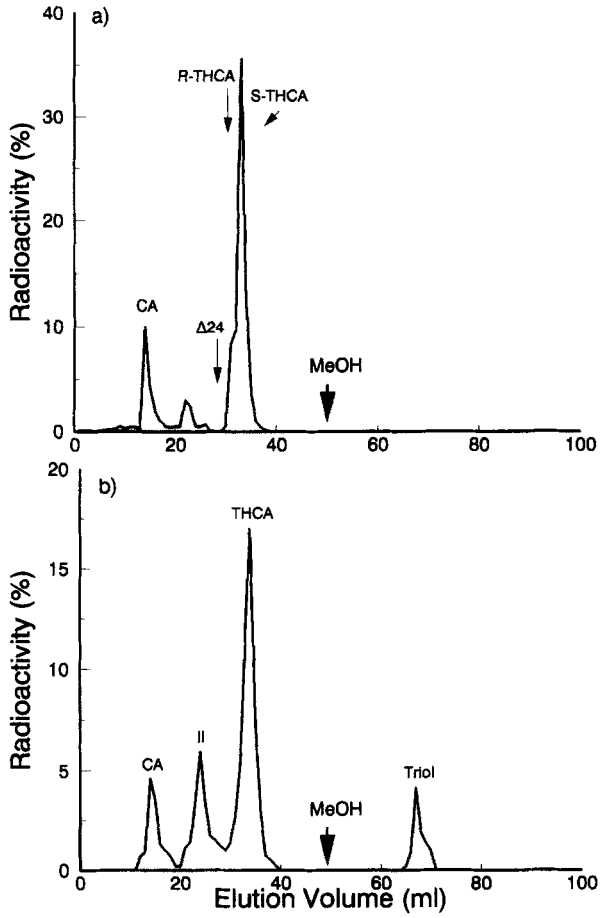

Fig. 3. HPLC profiles of a) ethyl acetate-extracted HepG2 cells incubated with 50 μg THCA for 96 h, and b) ethyl acetate extracts of the combined medium and HepG2 cells incubated with 50 μg 5β-cholestane-3α,7α,12α-triol for 24 h. The elution solvents were 24% of 30 mM TFA at pH 2.9 in methanol for 50 fractions when the solvent was changed to 100% methanol (indicated with an arrow marked MeOH) in both figures. Approximately 10,000 cpm was injected onto the column, which had a recovery of 90–100%. In a) the two configurations, R and S, of THCA (3α,7α,12α-trihydroxy-5β-cholestanoic acid) are marked. Cholic acid is marked CA and the position of the intermediate Δ24-THCA is indicated. In b) 5β-cholestane-3α,7α,12α-triol is marked triol, 3α,7α,12α-trihydroxy-5β-cholestanoic acid is THCA, and cholic acid is CA. The peak marked II is unidentified.

ability of the 7800 C1 Morris cells to form cholic acid, the cells were cultured in the presence of 13.3 μM THCA for 7 days or with 0.25 mM clofibric acid, 10 μM retinoic acid, 30 μM retinol, 250 nM dexamethasone, or 0.3 mM of two fatty acids, palmitic acid and linoleic acid, for 3 days (**Table 3**). None of these treatments restored the enzyme activities. All the treatments, except for those with THCA and linoleic acid, had an effect on the acyl-CoA oxidase activity.

Cholesterol 7α-hydroxylase activity in cell fractions from rat hepatocytes and HepG2 cells

In order to examine whether other enzymes involved in bile acid synthesis could be detected in cell extracts, the cholesterol 7α-hydroxylase activity was determined. The cholesterol 7α-hydroxylase activity in freshly prepared hepatocytes was lower than in rat microsomes, 1.20 and 1.70 nmol h^{-1} mg^{-1}, respectively. These results are in the same range as those found by other laboratories (31, 36). HepG2 cells had a much lower activity, although clearly detectable, 0.2 nmol h^{-1} mg^{-1}, which is 17% of that found in rat hepatocytes.

DISCUSSION

Our work demonstrates that human HepG2 cells have a defect in the oxidative cleavage of the C_{27} steroid side chain involved in bile acid synthesis. Two enzyme activities important for the oxidation of the bile acid intermedi-

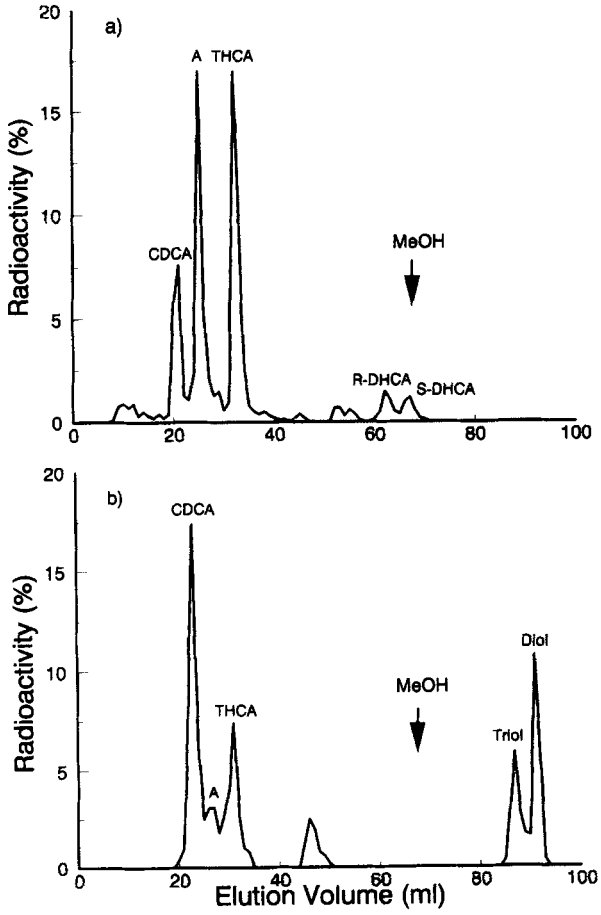

Fig. 4. HPLC profiles of ethyl acetate extracted media from incubations with a) 20 μg DHCA for 96 h, and b) 50 μg 5β-cholestane-3α,7α-diol for 24 h. The elution solvents were 24% of 30 mM TFA at pH 2.9 in methanol for 70 fractions when the solvent was changed to 100% methanol (indicated with an arrow marked MeOH) in both figures. Approximately 10,000 cpm was injected onto the column and the recovery was between 90 and 100%. In a) the R and S configurations of DHCA (3α,7α-dihydroxy-5β-cholestanoic acid) are marked and chenodeoxycholic acid is marked CDCA. In b) 5β-cholestane-3α,7α-diol is marked diol, 5β-cholestane-3α,7α,12α-triol is triol, 3α,7α,12α-trihydroxy-5β-cholestanoic acid is THCA, chenodeoxycholic acid is CDCA, and an unidentified peak is marked A.

TABLE 3. The effect of THCA, clofibric acid, retinoids, dexamethasone, and fatty acids on the acyl-CoA oxidase activity and the oxidation rate of THCA

	Acyl-CoA Oxidase	CA (1)[a]	CA (2)[b]
	nmol min^{-1} mg^{-1}	*nmol h^{-1} mg^{-1}*	
Control	2.75–2.86	n.d.	n.d.
THCA (13 μM)	2.80–2.88	n.d.	n.d.
Clofibric acid (0.25 mM)	10.64–9.84	n.d.	n.d.
Retinoic acid (10 μM)	5.73–6.52	n.d.	n.d.
Retinol (30 μM)	5.42–6.14	n.d.	n.d.
Dexamethasone (250 nM)	6.50–6.46	n.d.	n.d.
Palmitic acid (0.3 mM)	4.13–3.78	n.d.	n.d.
Linoleic acid (0.3 mM)	2.47–3.33	n.d.	n.d.

The enzyme activities were determined in 7800 C1 Morris rat hepatoma cells. The formation of cholic acid (CA) was measured. The values represent the activity obtained in duplicate cultures; n.d., not detectable.

[a] THCA alone.

[b] THCA and THCA-CoA ligase preparation.

ates THCA and DHCA appear to be deficient: the microsomal THCA-CoA ligase and the peroxisomal THCA-CoA oxidase. We find that another peroxisomal function is still intact in HepG2 cells, the peroxisomal β-oxidation of fatty acids, which agrees with other reports (19, 20). It has also been shown that HepG2 cells have several other peroxisomal activities (22). Similarly, we also show that a rat hepatoma cell line, 7800 C1 Morris cells, has the same defects in the oxidation of THCA and DHCA. The peroxisomal β-oxidation of fatty acids in 7800 C1 Morris cells is induced by peroxisome proliferators, such as clofibric acid and sulfur-substituted fatty acids (21, 22), which shows that these cells respond as normal cells in this respect.

The dramatic drop in the oxidation rate of THCA in rat hepatocytes with culture age compared to acyl-CoA oxidase activity also indicates that the enzyme activities involved in the formation of bile acids are easily lost. The THCA-CoA ligase and the THCA-CoA oxidase are distinct from the various acyl-CoA ligases (37) and the acyl-CoA oxidase (38). Thus, it is possible to have an intact peroxisomal β-oxidation of fatty acids, even if these enzyme activities involved in THCA oxidation are defective.

The findings presented here that cell extracts of HepG2 cells and 7800 C1 Morris cells could form cholic acid from Δ24-THCA-CoA, the product of the THCA-CoA oxidase, or from Δ24-THCA in the presence of the THCA-CoA ligase mean that the subsequent enzymes in the oxidation of THCA are active. We have previously shown that the enriched preparation of THCA-CoA ligase activates Δ24-THCA as efficiently as THCA (27). These findings support the conclusion, based upon analysis of metabolites found in plasma from patients with peroxisomal disorders (39, 40), and photoaffinity labeling peroxisomal enzymes with bile acid analogues (41), that the enoyl-CoA hydratase/3-hydroxy-acyl-CoA dehydrogenase (the trifunctional enzyme) and the 3-oxo-acyl-CoA thiolase (42) are shared between the peroxisomal β-oxidation of fatty acids and the oxidation of THCA. The different ratios between the intermediate 24-OH-THCA and cholic acid found in HepG2 cells and 7800 C1 Morris cells could be caused by differences in the stability of the thiolase (see Fig. 1), in these two species.

We also show that the oxidation of DHCA to chenodeoxycholic acid is lacking in cell extracts from HepG2 cells and 7800 C1 Morris cells. Similar to the oxidation of THCA, oxidation of DHCA has been reported to occur in the peroxisomes in the rat and humans (15, 16), most likely using the same enzyme pathway as THCA (43). The lack of formation of both cholic acid and chenodeoxycholic acid by cell extracts in the present study supports these suggestions. However, the low ability of HepG2 cell extract to convert the supposed Δ24-DHCA to chenodeoxycholic acid could mean that DHCA and intermediates thereof are not preferred substrates for the peroxisomal oxidative cleavage of the C_{27} steroid side chain in humans and therefore less efficiently oxidized. It has been suggested that 7α,27-dihydroxy-4-cholesten-3-one is the preferred substrate in chenodeoxycholic acid formation (12, 44).

Several studies investigating bile acid formation de novo in HepG2 cells have used cell cultures incubated for 72–96 h. The first and rate-limiting enzyme in bile acid synthesis, cholesterol 7α-hydroxylase, is known to have an enzyme activity that decreases with culture age in hepatocyte primary cultures. It is maintained by certain hormones, such as dexamethasone and T_3 (36, 45). This activity is low in these cells and we determined it to be approximately 17% of the activity found in rat hepatocytes. As these cells have been found to secrete low amounts of cholic acid into the medium compared to chenodeoxycholic acid and even secrete THCA, it has been suggested that they have an impaired peroxisomal oxidation of THCA (8). Similar results were obtained in the present study with cells incubated with 5β-cholestane-3α,7α,12α-triol. The main product of 5β-

cholestane-3α,7α,12α-triol was THCA that was accumulated, whereas cholic acid was found only in a relatively small amount. One possible explanation for these results is that the peroxisomal oxidation is working, but at an extremely low rate. Cholic acid was also formed by the cells in 96-h incubations with THCA as substrate. In ethyl acetate extracts from the cells, however, no Δ24-THCA was found. This lack of Δ24-THCA suggests that the cholic acid secreted into the medium results from alternative pathways, such as the microsomal 25-hydroxylase pathway (for review, see ref. 46). It is not reported, however, that THCA is a substrate for this pathway.

The predominant preliminary bile acid formed by HepG2 cells in previous reports was chenodeoxycholic acid (8–10). This bile acid was also found in the present investigation when the cells were incubated with 5β-cholestane-3α,7α-diol or DHCA. Ethyl acetate extracts of the medium from incubations with DHCA gave, in addition to the chenodeoxycholic acid peak, two other major peaks on HPLC. One corresponded to THCA and the other to a peak previously found in incubations with L-fractions from rats (16). It is not known whether this is an intermediate of the peroxisomal degradation of DHCA. Ethyl acetate extracts from the combined cell and medium from incubations with 5β-cholestane-3α,7α-diol did not have an accumulation of DHCA; instead relatively large peaks of 5β-cholestane-3α,7α,12α-triol and THCA appeared. Cholic acid could not be detected in this extract, which further emphasizes the suggestion of an impaired oxidation of THCA. Similar to the cholic acid formation, alternative pathways for chenodeoxycholic acid formation in rat and man do exist (for review, see ref. 46), such as the mitochondrial 27-hydroxylation of cholesterol. In addition, there are reports that the mitochondrial fraction (15, 47) and the microsomal fraction (15, 48) can contribute to the formation of chenodeoxycholic acid. The chenodeoxycholic acid observed in long-term incubations could thus be formed by low capacity alternative pathways, which we were unable to detect during a short incubation period with cell extract. In a recent study using HepG2 cells, chenodeoxycholic acid and cholic acid were synthesized in equal amounts and no evidence was found for the existence of the alternative pathway starting with 27-hydroxylation of cholesterol (12). It cannot, therefore, be excluded that differences in cell populations from the same cell line exist.

In summary, we have shown that enzymes involved in the oxidation of THCA and DHCA, the microsomal THCA-CoA ligase and the peroxisomal THCA-CoA oxidase, appear to have been lost or impaired during the transformation process to these hepatoma cells. Attempts to induce the defective enzyme activities in 7800 C1 Morris cells by adding a number of agents, including drugs and hormones, such as THCA, fatty acids, clofibric acid, retinoic acid, or dexamethasone, to the culture media failed. This indicates that the THCA-CoA ligase and the THCA-CoA oxidase activities in 7800 C1 Morris cells are irreversibly impaired. These findings of defective peroxisomal enzyme activities suggest that results obtained from studies performed on hepatoma cell lines, such as the often used HepG2 cells (9–12), must be interpreted with caution as to normal formation and regulation of primary bile acids. Furthermore, we show that the subsequent peroxisomal enzymes needed to obtain the primary bile acids cholic acid and chenodeoxycholic acid are not lost. These findings support the suggestion that the trifunctional enzyme and the thiolase are shared between the oxidation of THCA, and the peroxisomal β-oxidation of fatty acids and that THCA and DHCA are oxidized by the same enzyme system.

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