Hepatic 7α-dehydroxylation of bile acid intermediates, and its significance for the pathogenesis of cerebrotendinous xanthomatosis

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Abstract The bile acid precursor 7α-hydroxy-4-cholesten-3-one was found to be enzymatically dehydroxylated at a slow rate by liver tissues from the rat, human, and guinea pig. The rat liver enzyme is localized in the microsomal fraction, has a pH optimum of about 8.5, an apparent K_m of 0.03–0.04 mM, and a V_{max} of 10–15 nmoles · mg protein^{-1} · hr^{-1}. The product from 7α-hydroxy-4-cholesten-3-one was identified as cholesta-4,6-dien-3-one by its chromatographic properties and by mass spectrometry. The reaction proceeded both in air and N_2, and pyridine nucleotides were not required as cofactors. In addition to the enzymatic reaction, there was a significant nonenzymatic dehydroxylation of 7α-hydroxy-4-cholesten-3-one, in particular at high pH and with high concentrations of protein. No 7α-dehydroxylation occurred with various 7α-hydroxylated 3β-hydroxy-Δ5-steroids. We have previously shown that at least part of the accumulation of cholestanol in cerebrotendinous xanthomatosis (CTX) is due to accelerated 7α-dehydroxylation of bile acid intermediate(s), which are further converted into cholestanol. The capacity to dehydroxylate 7α-hydroxy-4-cholesten-3-one was found to be about the same in homogenates of liver biopsies from two patients with CTX as in preparations from control subjects. It is suggested that increased levels of substrate (7α-hydroxy-4-cholesten-3-one) in the liver, rather than increased amounts of 7α-dehydroxylase is the explanation for the accelerated 7α-dehydroxylation in CTX that leads to increased biosynthesis of cholestanol. — **Skrede, S., M. S. Buchman, and I. Björkhem.** Hepatic 7α-dehydroxylation of bile acid intermediates, and its significance for the pathogenesis of cerebrotendinous xanthomatosis. *J. Lipid Res.* 1988. **29:** 157–164.

Supplementary key words 7α-hydroxy-4-cholesten-3-one • cholesta-4,6-dien-3-one • cholestanol • 26-hydroxylase deficiency

The main symptoms in the rare inherited disease cerebrotendinous xanthomatosis (CTX) are caused by accumulation of cholestanol (1). The mechanism of the accelerated biosynthesis of cholestanol in this disease (2) has not been clarified in detail. The primary enzymatic defect in CTX is a deficiency of a mitochondrial steroid 26-hydroxylase involved in the main pathway for formation of bile acids from cholesterol (3, 4), and it is difficult to understand how this defect can lead to increased biosynthesis of cholestanol. The only previously known pathway for biosynthesis of cholestanol involves a direct conversion of cholesterol through 4-cholesten-3-one (5–8). We have shown, however, that the primary block in CTX causes accumulation of 7α-hydroxy-4-cholesten-3-one and other bile acid intermediates in the liver (9), and that cholestanol can be formed from 7α-hydroxy-4-cholesten-3-one through a novel pathway (10–12). We have recently presented evidence that this pathway is accelerated in CTX (12).

In order to serve as precursor for cholestanol, 7α-hydroxy-4-cholesten-3-one must lose its 7α-hydroxyl group. According to results of different experiments in vivo, the major part of the dehydroxylation of 7α-hydroxy-4-cholesten-3-one seems to occur in the liver (11–13).

In the present work we have studied enzymatic 7α-dehydroxylation of early bile acid intermediates in preparations from mammalian liver.

MATERIALS AND METHODS

Materials

[4-^{14}C]-7α-Hydroxy-4-cholesten-3-one and [7β-^{3}H]-7α-hydroxy-4-cholesten-3-one were synthesized as described previously (10, 14), and [7β-^{3}H]-7α-hydroxycholesterol was synthesized according to Danielsson and Einarsson (15). [7α-^{3}H]-7β-Hydroxycholesterol was synthesized as described previously (14).

Abbreviation: CTX, cerebrotendinous xanthomatosis.

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Patients

Needle biopsies of liver tissue were obtained from two sisters with cerebrotendinous xanthomatosis (A. F., born 27 July 1941 and I. J., born 6 May 1935). Their records have been described previously (4, 12, 16). Control biopsies of normal human liver were obtained from three patients who underwent abdominal surgery for aortic aneurysm (Control 1: male, 69 yr), cancer cardiae without liver metastases (Control 2: male, 73 yr), and hepatic hemangioma (Control 3: female, 23 yr).

Animals

Male Wistar rats weighing about 200 g were used in most animal studies. Male guinea pigs weighing 500–600 g were used in a few experiments.

Preparation of tissue subfractions, marker enzymes

For the studies of the subcellular localization of the enzymatic dehydroxylation of 7α-hydroxy-4-cholesten-3-one, subfractions of rat liver were prepared essentially according to de Duve and coworkers (17) with the modifications described previously (18). Microsomal fractions were prepared by differential centrifugation from 1:5 (w/v) homogenates in 0.25 M sucrose from the liver after removal of the "nuclear" fraction at 30,000 *g*-min and the combined mitochondrial and lysosomal fractions at 250,000 *g*-min. The microsomal pellet was obtained by centrifugation at 3,000,000 *g*-min. Marker enzymes were assayed as previously described (18, 19).

Dehydroxylation of bile acid intermediates

In standard incubations, labeled 7α-hydroxy-4-cholesten-3-one or other intermediates, 20 μg dissolved in 10 μl of acetone, were added to 100–150 μg of microsomal protein in a total volume of 500 μl of 0.05 M Tris-Cl buffer, pH 8.5. The incubations were performed for 1 hr at 37°C. A corresponding blank incubation was always performed simultaneously, using the same amount of microsomes treated for 12 min at 80°C. The enzymatic conversion was calculated as the difference between the conversion obtained with untreated and heat-activated microsomes. In some experiments the incubation conditions were designed to avoid the initial lag in the dehydroxylation reaction. In these experiments incubations with both untreated and heat-inactivated microsomes were performed both for 40 min and for 100 min. The enzymatic conversion during the latter 60-min incubation period (during which there is a linear relation between conversion and incubation time) was calculated as the difference between the enzymatic (corrected) conversion obtained after 100 min of incubation and the corresponding (corrected) conversion after 40 min.

The incubations were stopped by adding 2 ml of ethanol followed by 3 ml of water. Extractions were performed twice with n-hexane–ethyl acetate 1:1 (v/v).

In most experiments, about 0.2 μCi of [7β-^{3}H]-7α-hydroxy-4-cholesten-3-one was used in each incubation. In some experiments in which radioscanning of the chromatoplates was performed, about 2 μCi of [7β-^{3}H]-7α-hydroxy-4-cholesten-3-one was used.

Thin-layer chromatography and measurement of radioactivity

Extracts obtained as described above were redissolved in toluene–ethyl acetate 1:1 (v/v). This was also the mobile phase for thin-layer chromatography that was performed on glass plates coated with Silica gel-H. In this system R_f values were as follows: 7α-hydroxycholesterol, 0.21; 7α-hydroxy-4-cholesten-3-one, 0.45; and cholesta-4,6-dien-3-one, 0.68. In the standard assay of enzymatic 7α-dehydroxylation, 0.5-cm zones of the gel were scraped into counting vials, 10 ml of Packard Insta Gel II (Packard Instrument Co., Inc., Downers Grove, IL) was added, and radioactivity was measured with a Packard Tri-Carb scintillation spectrometer, model 3385. In some experiments (see Fig. 2) thin-layer chromatoplates were subjected to radioscanning, using an instrument from Berthold (Karlsruhe, West Germany).

High performance liquid chromatography and mass spectrometry

These were performed under the conditions described previously (11, 12).

RESULTS

Subcellular location of 7α-dehydroxylase activity

Fig. 1 shows the results of one typical subfractionation experiment with rat liver. The specific activities of the marker enzymes were highest in the fractions expected: carnitine palmitoyltransferase (A) in the "mitochondrial" fraction, catalase (B) in the "lysosomal" and "mitochondrial" fractions, acid phosphatase (C) in the "lysosomal" fraction, and glucose-6-phosphatase (E) in the "microsomal" fraction. The plasma membrane marker phosphodiesterase I (F) showed a typical "nucleo-microsomal" distribution, as previously observed (19).

It is evident that the highest enzymatic capacity for removal of the 7α-hydroxyl group of 7α-hydroxy-4-cholesten-3-one (D) resides in the microsomal fraction. The mitocondrial fraction also has a rather high activity. The subcellular distribution of this activity is similar to that of glucose-6-phosphatase (E).

Identification of product

Fig. 2 shows that the product of the 7α-dehydroxylation of 7α-hydroxy-4-cholesten-3-one had the same thin-layer chromatographic properties as cholesta-4,6-dien-3-one. In addition, the product had the same R_f (27

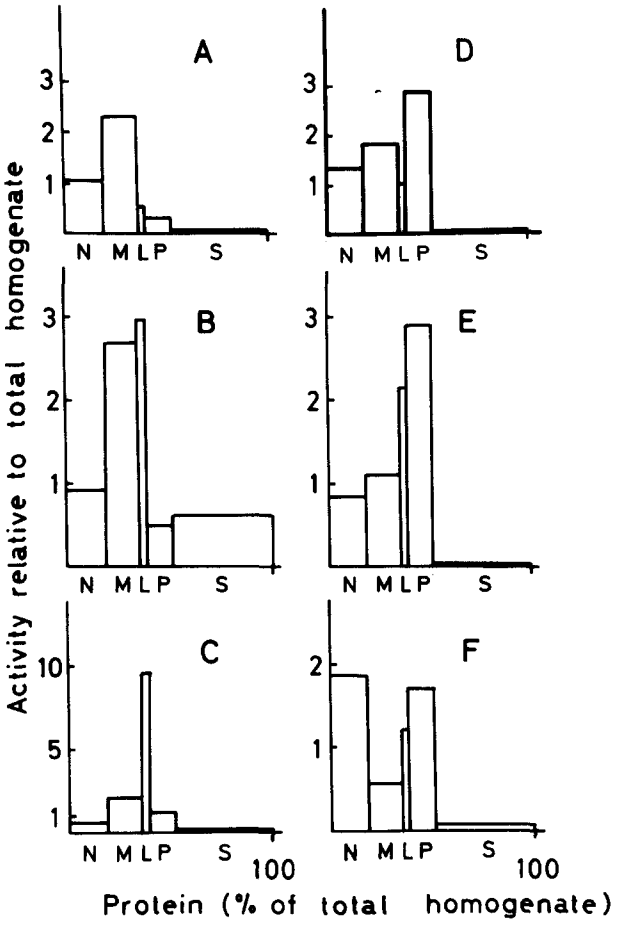

Fig. 1. Subcellular localization of 7α-hydroxysteroid dehydroxylase in rat liver, as compared with marker enzymes. Subfractions of rat liver were prepared in sucrose (0.25 mmol/l) without additions, and the enzymes were assayed as described in Materials and Methods. The specific activities of the enzymes in the subfractions expressed per mg of protein were compared with those of the total homogenate (= 1 on the ordinate), which were as follows: A, carnitine palmitoyl transferase, 1450 dpm · min^{-1} · mg^{-1}; B, catalase, 51.2 mmol · mg^{-1} · min^{-1}; C, acid phosphatase, 20 nmol · mg^{-1} · min^{-1}; D, 7α-hydroxysteroid dehydroxylase, 5.6 nmol · mg^{-1} · h^{-1}; E, glucose-6-phosphatase, 86 nmol · mg^{-1} · min^{-1}; F, phosphodiesterase I, 99 nmol · mg^{-1} · min^{-1}. The fractions were: N, nuclear; M, mitochondrial; L, lysosomal; P, microsomal; S, supernatant.

min) on high performance liquid chromatography as authentic cholesta-4,6-dien-3-one. Furthermore, the mass spectrum of the product from 7α-hydroxy-4-cholesten-3-one was identical with that of the standard (**Fig. 3**).

Assay conditions for 7α-dehydroxylation of 7α-hydroxy-4-cholesten-3-one

The pH optimum was found to be about 8.5 for the dehydroxylation of 7α-hydroxy-4-cholesten-3-one by rat liver microsomes (**Fig. 4**). Above this pH, there was a high nonenzymatic 7α-dehydroxylation with a decreasing enzymatic reaction. The nonenzymatic 7α-dehydroxylation was accelerated by the addition of microsomes inactivated at 80°C for 12 min. EDTA was without effect on the enzymatic 7α-dehydroxylation of 7α-hydroxy-4-cholesten-3-one, and also on the catalysis by boiled microsomes. In the following experiments, 0.05 M Tris-Cl, pH 8.5, was used as incubation medium and the nonenzymatic conversion was corrected with the use of heat-inactivated microsomes. Several sets of experiments were repeated also with 0.05 M potassium phosphate buffer, pH 7.4, instead of 0.05 M Tris-Cl buffer, pH 8.5. With 0.05 M potassium phosphate buffer, the enzymatic conversion was somewhat lower and this was also the case with the nonenzymatic conversion. Use of the two different buffers gave essentially the same results otherwise.

Fig. 5A shows that the time course of the enzymatic dehydroxylation of 7α-hydroxy-4-cholesten-3-one by rat liver microsomes corrected for nonenzymatic conversion had a definite lag period, with a slow activity during the first 30–40 min of incubation. Thereafter, the reaction proceeded linearly until 2 hr. In separate experiments (not shown) the reaction was linear with time between 40 and 180 min of incubation but leveled off after 240 min. Several experiments similar to that shown in Fig. 5A were performed and it was ascertained that the lag period was a constant and reproducible finding.

Fig. 5B shows the dependency of the enzymatic 7α-dehydroxylation of 7α-hydroxy-4-cholesten-3-one on the amount of microsomal protein. The activity increased linearly up to about 150 μg of microsomal protein, and then leveled off quite abruptly. The reaction rate was equal in air and nitrogen atmosphere, and was not altered by the addition of NAD, NADH, NADP, or NADPH. Why the velocity of the reaction declined at higher concentrations of microsomal protein could not be clarified. Addition of unlabeled cholesta-4,6-dien-3-one, the product of the dehydroxylation, caused a slight inhibition of the dehydroxylation of 7α-hydroxy-4-cholesten-3-one only when present in great excess. At 100 μM of 7α-hydroxy-4-

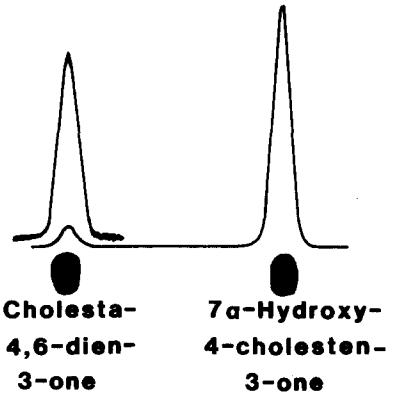

Fig. 2. Thin-layer radiochromatogram of the substrate and product isolated after incubation of [7β-^{3}H]7α-hydroxy-4-cholesten-3-one with rat liver microsomes under standard incubation conditions. The sensitivity in the recording of the upper tracing was about tenfold higher than that in the recording of the lower tracing.

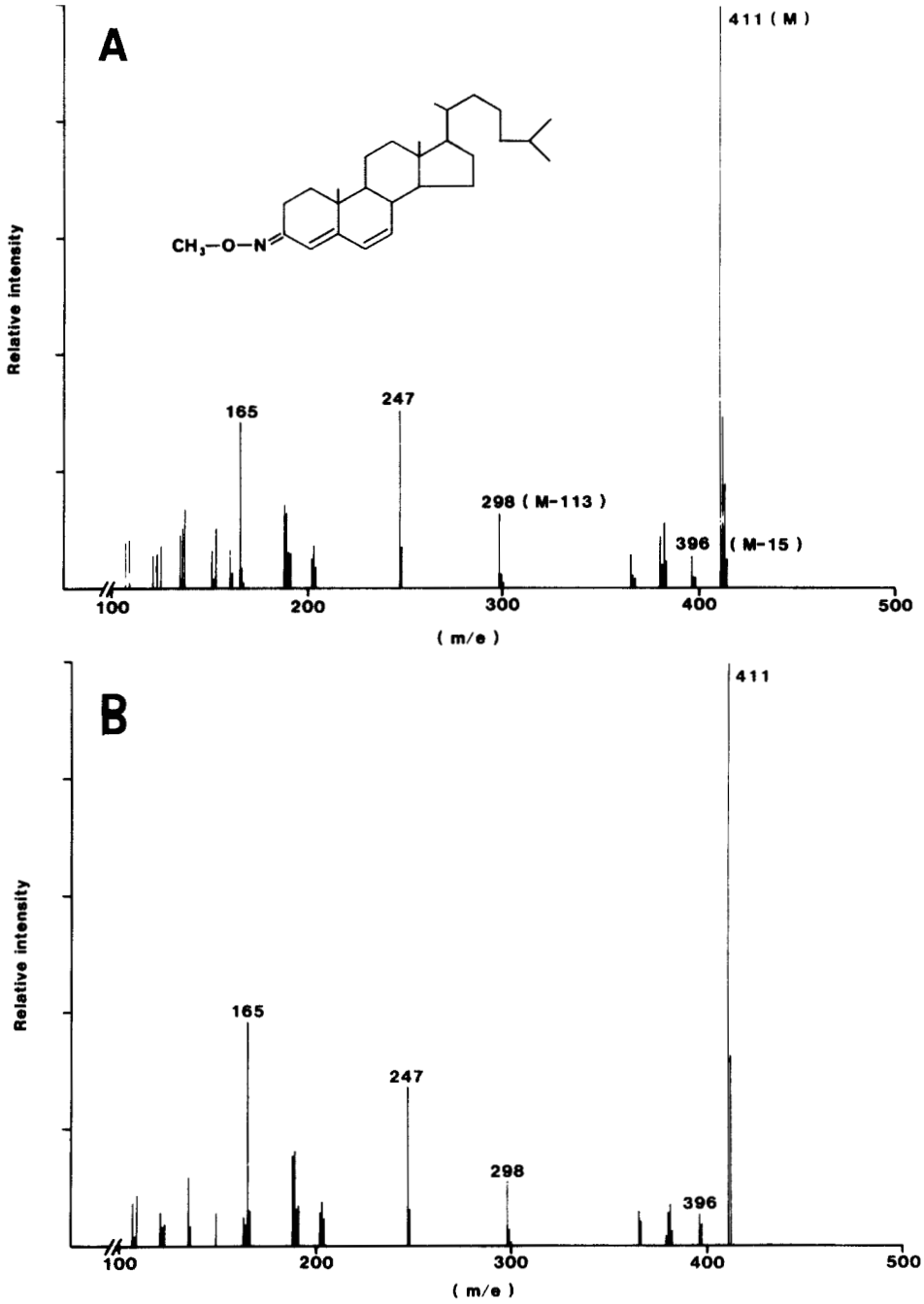

Fig. 3. Mass spectrum of cholesta-4,6-dien-3-one formed after incubation of 7α-hydroxy-4-cholesten-3-one with rat liver microsomes. A: Mass spectrum of the methoxime of the reference compound, cholesta-4,6-dien-3-one. B: Mass spectrum of the methoxime of the product from 7α-hydroxy-4-cholesten-3-one incubated with rat liver microsomes in the absence of NAD or other cofactors (cf. Materials and Methods). The product was isolated by thin-layer chromatography as shown in Fig. 2.

cholesten-3-one (as used in the experiment shown by Fig. 5), the inhibition by 100 μM of cholesta-4,6-dien-3-one was only 9%. Addition of unlabeled 4-cholesten-3-one, 5α-cholestan-3-one, 7α-hydroxycholesterol, or cholic acid did not inhibit the enzymatic dehydroxylation of 7α-hydroxy-4-cholesten-3-one. The nonenzymatic dehydroxylation of 7α-hydroxy-4-cholesten-3-one was quite insignificant with low amounts of heat-inactivated microsomes (less than 50 μg), but accounted for almost 50% of the 7α-dehydroxylation at high microsomal protein concentrations (above 200 μg). The possibility must be considered that there was an incomplete inactivation of the enzyme under the conditions used.

Fig. 5C shows the effect of the substrate concentration

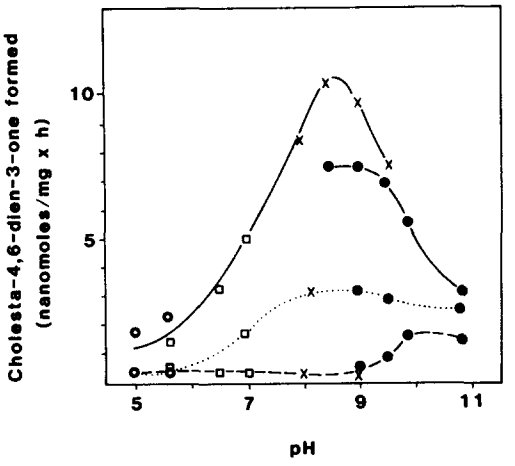

Fig. 4. Dependency on pH of the dehydroxylation of 7α-hydroxy-4-cholesten-3-one by rat liver microsomes. 7α-Hydroxy-4-cholesten-3-one (0.1 mM) was incubated with rat liver microsomes (0.15 mg of protein) for 1 hr at 37°C. The following buffers were added at a concentration of 0.05 M, giving a final volume of 500 μl: acetate buffer (○); phosphate buffer (□); Tris-buffer (X); glycine buffer (●). Unbroken curve, active microsomes present; stippled curve, heat-inactivated microsomes (80°C, 12 min.) present; broken curve, microsomes not added.

on the enzymatic dehydroxylation of 7α-hydroxy-4-cholesten-3-one with rat liver microsomes. In the experiment shown in Fig. 5C, a low amount (50 μg) of microsomal protein was used, but similar results were obtained with 150 μg. With high concentration of protein (1.2 mg, results not shown) no substrate saturation was obtained. By using a computerized version of Clelands enzyme kinetic program (20), the apparent K_m of the enzymatic dehydroxylation of 7α-hydroxy-4-cholesten-3-one with rat liver microsomes was estimated to be about 0.03 mM and V_{max} to be about 13 $nmol \cdot mg^{-1} \cdot hr^{-1}$.

Several difficulties thus appeared in the attempts to define the kinetics of 7α-dehydroxylation of 7α-hydroxy-4-cholesten-3-one: an initial unexplained lag period of the reaction, slight product inhibition, and linearity only at relatively low concentrations of microsomal protein. The most disturbing factor was the lag of the reaction. The possibility that this lag period was caused by endogenous microsomal lipids was investigated by studying 7α-dehydroxylation of 7α-hydroxy-4-cholesten-3-one in acetone powder preparations of rat liver microsomes (21). Such preparations had about the same specific activity towards 7α-hydroxy-4-cholesten-3-one as crude microsomes, and the activity was linear with the amount of acetone powder. However, also with acetone powder, a lag of the reaction was observed similar to that found in crude microsomes (results not shown). The possibility that the lag period was caused by an inhibitor which is destroyed after the first 30–40 min of incubation was also tested by preincubation of the microsomal fraction for 45 min prior to addition of the substrate. With these preincubated microsomes also, there was a lag period similar to that shown in Fig. 5A. The lag period was obtained also in experiments where 100,000 *g* supernatant had been added to the incubation mixture. From a theoretical point of view, the most accurate condition for assay of enzymatic activity should be in the time interval between 40 and 100

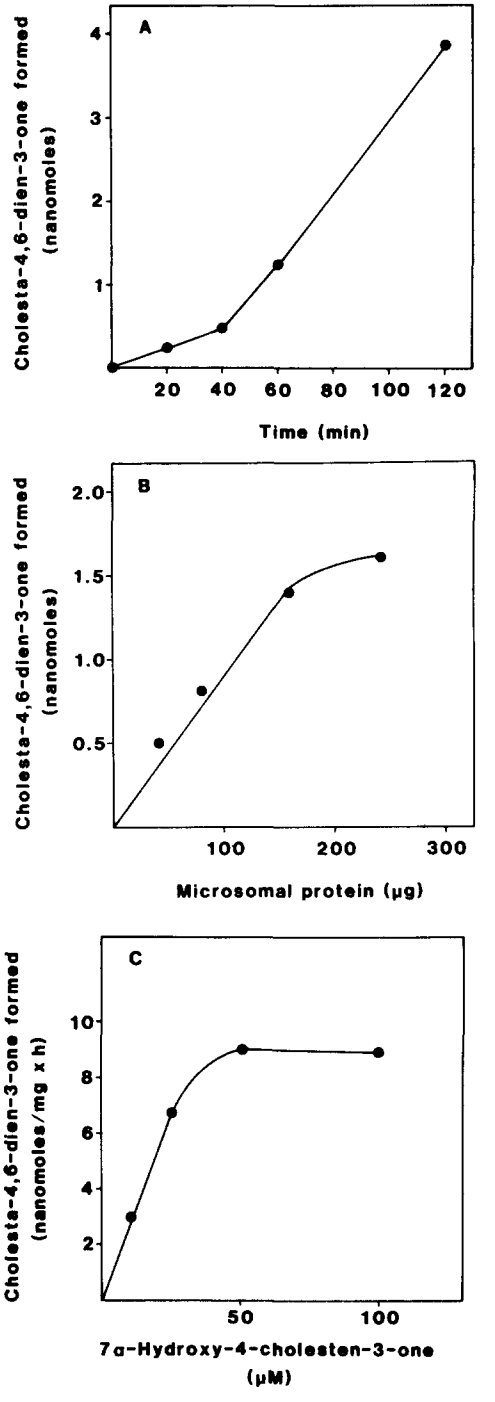

Fig. 5. Effect of time (A), enzyme concentration (B), and substrate concentration (C) on enzymatic 7α-dehydroxylation of 7α-hydroxy-4-cholesten-3-one. Except for the varied parameters, standard incubation conditions were used.

min of incubation. In a series of experiments, incubations were therefore performed both for 40 min and 100 min (together with appropriate control incubations with boiled microsomes). The enzymatic conversion after 100 min minus the enzymatic conversion after 40 min was calculated. This conversion was shown to be linear with microsomal protein and it was possible to saturate the enzyme with substrate. In the latter experiment, the K_m was somewhat higher (about 0.04 mM) than that obtained in the above experiments.

The V_{max} values obtained in these experiments were similar to those obtained under standard incubation conditions.

Substrate specificity

The following 7α- and 7β-hydroxylated steroids could not serve as substrate for the enzyme: 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 24-ethyl-5-cholesten-3β,7α-diol. Except for 7β-hydroxycholesterol, these substrates could be converted to the corresponding Δ^6-products after addition of NAD to the incubation mixture. In these cases, 7α-hydroxy-4-cholesten-3-one and 24-ethyl-7α-hydroxy-4-cholesten-3-one, respectively, were intermediates in the reactions.

Experiments with preparations from human and guinea pig liver

Table 1 shows the rate of enzymatic dehydroxylation of 7α-hydroxy-4-cholesten-3-one by total homogenates of human liver (using standard incubation conditions). Tissue concentration curves (not shown) established that there was a linear relationship between the amounts of tissue and the conversion. This is also evident from the parallel experiments, run at different protein concentrations (Table 1). If it is assumed that microsomal protein constitutes about 10% of the total protein of the homogenates, the specific activities of the enzymatic 7α-dehydroxylation in human liver appeared to be considerably higher than in rat liver (V_{max} in rat liver microsomes was calculated to be about 13 nmol · mg^{-1} · hr^{-1}). It is evident from the table that the capacity for 7α-dehydroxylation in liver biopsy homogenates from two patients with cerebrotendinous xanthomatosis was not different from that of the controls.

Some experiments were also performed with microsomal preparations from guinea pigs. The enzymatic capacity of these preparations was similar to that of rat liver microsomes (results not shown).

TABLE 1. Dehydroxylation of 7α-hydroxy-4-cholesten-3-one in homogenates of liver biopsies from human controls and two patients with cerebrotendinous xanthomatosis

Subject	Protein	Cholesta-4,6-dien-3-one Formed
	mg	*nmol · mg protein^{-1} · hr^{-1}*
Control 1	0.11	4.0
Control 1	0.22	4.6
Control 2	0.09	1.5
Control 2	0.18	1.6
Control 3	0.08	2.1
Control 3	0.16	1.5
CTX subject I. J.	0.09	2.4
CTX subject I. J.	0.19	3.2
CTX subject A. F.	0.08	2.8
CTX subject A. F.	0.16	2.9

Human liver biopsies (see Methods section) werre homogenized in Tris-buffer (50 mM, pH 9.0) and incubated with 7α-hydroxy-4-cholesten-3-one (100 μM) for 1 hr at 37°C in Tris-buffer (50 mM, pH 9.0). The conversion in active homogenate is subtracted from the conversion in the blank incubation.

DISCUSSION

In this study, evidence is presented for a previously unrecognized enzymatic activity in mammalian liver, causing the removal of the 7α-hydroxyl group of 7α-hydroxy-4-cholesten-3-one. The product was identified as cholesta-4,6-dien-3-one. Results of preliminary experiments in our laboratory using [6β-^{3}H]-7α-hydroxy-4-cholesten-3-one (14), indicate that the mechanism mainly involves a *trans* diaxial elimination of the 7α-hydroxyl group and the 6β-hydrogen. A similar mechanism has been described for the formation of secondary bile acids by intestinal microorganisms (22). The elimination of water from 7α-hydroxy-4-cholesten-3-one should be facilitated by the 3-oxo-Δ^4-structure, due to the energetic properties of the conjugated product formed. A similar dehydroxylation of 7α-hydroxy-4-cholesten-3-one can be obtained nonenzymatically by treatment with $POCl_3$ or weak alkali (14) and a significant nonenzymatic 7α-dehydroxylation was obtained in the present experiments especially when pH was higher than 8 and after addition of heat-inactivated microsomes. The enzymatic reaction had a pH optimum about 8.5, was independent of cofactors, and proceeded with equal rate in air and nitrogen atmosphere. The kinetics of the reaction was difficult to define due to the limited solubility of the substrate, a slight product inhibition, an initial lag period of the reaction, and a lack of linearity with high concentrations of microsomes. The latter phenomenon may be due to the presence of inhibitors in the preparations.

To our knowledge, the present enzyme has not been described previously. Slaytor and Bloch (23) have shown the conversion of Δ^7-cholestene-3β,6β-diol to cholesterol by rat liver homogenates, but this reaction proceeded only under aerobic conditions. An enzyme has been isolated from the soluble fraction of yeast, catalyzing the anaerobic dehydration of ergo-7,22-diene-3β,5α-diol (24). Topham and Gaylor (24) observed that the enzymatic dehydroxylation of this steroid declined rapidly with time. In contrast, we found a definite lag period for the dehydroxylation of

7α-hydroxy-4-cholesten-3-one by liver microsomes. The reason for this lag period could not be clarified. The lag period was not due to a simple substrate-independent removal of endogenous inhibitors since the lag period was present also in experiments with microsomes preincubated prior to addition of substrate.

Regardless of the problems in defining the kinetic properties of the present enzyme and the presence of a significant nonenzymatic conversion, the hepatic dehydroxylation of 7α-hydroxy-4-cholesten-3-one demonstrated here is of interest in relation to the accumulation of cholestanol in patients with CTX.

Mechanism of the increased biosynthesis of cholestanol in cerebrotendinous xanthomatosis

Cholestanol can be synthesized in the liver from cholesterol via 4-cholesten-3-one (11, 25, 26). In the hereditary disease cerebrotendinous xanthomatosis, the synthesis of cholestanol is increased (27), and the main symptoms are caused by sterol depositions in different tissues, particularly in the central nervous system. It has recently been proposed by Salen, Shefer, and Tint (28) that the formation of 4-cholesten-3-one from cholesterol is increased in CTX because of increased activity of the Δ^5-3β-hydroxysteroid dehydrogenase involved in bile acid biosynthesis. In a recent investigation, however, we found normal levels of this enzyme in liver biopsies from patients with cerebrotendinous xanthomatosis (29). The reason for the increased biosynthesis of cholestanol in CTX is, according to our work, an increased activity of a novel pathway involving 7α-hydroxy-4-cholesten-3-one (10–12).

Significance of hepatic 7α-dehydroxylation of 7α-hydroxy-4-cholesten-3-one for the pathogenesis of cerebrotendinous xanthomatosis

The primary enzymatic defect in cerebrotendinous xanthomatosis is a deficiency of a mitochondrial C-27 steroid 26-hydroxylase (3, 4). This causes the accumulation in the liver of intermediates of bile acid biosynthesis, among these 7α-hydroxy-4-cholesten-3-one (9). In normal human liver, the concentration of 7α-hydroxy-4-cholesten-3-one is about 5 ng/mg protein, i.e., approximately 2.5 μM. In the present study, we have shown that the K_m for 7α-hydroxy-4-cholesten-3-one of the rat liver 7α-dehydroxylase is about 0.03–0.04 mM. In CTX, the hepatic concentration of 7α-hydroxy-4-cholesten-3-one is increased about tenfold, approaching the K_m for the rat liver 7α-hydroxylase. This means that even when the concentration of the enzyme is not increased, as indicated in the present study, increased substrate concentrations might lead to increased 7α-dehydroxylation of 7α-hydroxy-4-cholesten-3-one. The product, cholesta-4,6-dien-3-one, can be converted in the liver to cholestanol via 4-cholesten-3-one (30). We have recently shown that the concentration of cholesta-4,6-dien-3-one in blood is increased in patients with CTX (13, 31). We have also recently presented evidence for increased utilization of this novel 7α-hydroxylation–dehydroxylation pathway in CTX by demonstrating increased loss of 7α-^{3}H-label during the conversion of cholesterol to cholestanol in this disease (12). Normally, this pathway contributes with less than ¼ of the biosynthesis of cholestanol (11, 12), and the "direct" route via 4-cholesten-3-one with ¾ or more (11, 12).

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