Cholic acid biosynthesis: conversion of 5β-cholestane-3α,7α,12α,25-tetrol into 5β-cholestane-3α,7α,12α,24β,25-pentol by human and rat liver microsomes

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Abstract This paper describes the conversion of 5β-cholestane-3α,7α,12α,25-tetrol into 5β-cholestane-3α,7α,12α,24β,25-pentol by liver microsomes. A sensitive radioactive assay for measuring the formation of 5β-cholestane-3α,7α,12α,24β,25-pentol was developed. Optimal assay conditions for human and rat microsomal systems were established. A higher 24β-hydroxylation activity was detected in rat than in human liver under the conditions employed. The hydroxylation of 5β-cholestane-3α,7α,12α,25-tetrol by the rat liver microsomal fraction fortified with NADPH was stimulated about two-fold by administration of phenobarbital. Phenobarbital treatment also stimulated hydroxylations at C-23, C-24α, and C-26. Carbon monoxide markedly inhibited all side-chain hydroxylations. In contrast, side-chain hydroxylase activities were not affected in animals deprived of food for 48 hr. These results are consistent with a previously postulated cholic acid biosynthetic pathway involving 5β-cholestane-3α,7α,12α,24β,25-pentol as a key intermediate in man and in the rat.

Supplementary key words cerebrotendinous xanthomatosis · 24β-hydroxylation · liver microsomal enzymes · bile acids · bile alcohols · biosynthetic pathway

5β-Cholestane-3α,7α,12α,25-tetrol is a key intermediate of cholic acid biosynthesis in patients with CTX (1). A 25-hydroxylation pathway of cholic acid synthesis was also shown to be present as an alternate pathway in normolipidemic subjects (2) and in the rat (3, 4).

It has previously been demonstrated by Cronholm and Johansson in the rat (4) and by Björkhem et al. in man (5) that the major product formed during the incubation of 5β-cholestane-3α,7α,12α-triol with liver microsomes was 5β-cholestane-3α,7α,12α,25-tetrol. It was further demonstrated in our laboratory (6) that, both in man and in the rat, liver microsomal enzymes catalyze hydroxylations of 5β-cholestane-3α,7α,12α,25-tetrol at C-23, C-24α, C-24β, and C-26. However, only 5β-cholestane-3α,7α,12α,24β,25-pentol, but not the other 5β-cholestanepentols, is rapidly transformed into cholic acid by soluble enzymes (6). According to the above report, the side-chain hydroxylation of 5β-cholestane-3α,7α,12α,25-tetrol required NADPH and O_2, which suggested the involvement of a mixed-function oxidase. In contrast, Masui and Staple (7) and, more recently, Gustafsson (8) postulated that in the biosynthetic pathway of cholic acid involving 26-oxygenated intermediates, the 24-hydroxylation step was not catalyzed by a mixed-function oxidase, but was analogous to fatty acid β-oxidation and involved an acyl dehydrogenase and enoyl hydrase.

In the present investigation the enzyme system catalyzing the conversion of 5β-cholestane-3α,7α,12α,25-tetrol to 5β-cholestane-3α,7α,12α,24β,25-pentol was studied in more detail. Optimal assay conditions were established both in man and in the rat, and the effects of phenobarbital treatment and carbon monoxide suggest that this microsomal system is probably a mixed-function oxidase involving the participation of cytochrome P-450.

MATERIALS

Clinical

These studies were conducted in four normolipidemic subjects with chronic peptic ulcer disease. The patients were hospitalized at the East Orange Veterans

Abbreviations: CTX, cerebrotendinous xanthomatosis; MS, mass spectrometry; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; TMSi, trimethylsilyl.

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Administration Hospital, and were fed regular hospital diets. Liver chemistries and blood coagulation tests were normal. Subtotal gastrectomies with vagotomies were performed on the subjects, and specimens of liver were obtained during surgery. After the induction of anesthesia with Enflurane, Ohio Medical Corp., Dayton, Ohio, the abdomen was opened and liver specimens weighing 2–4 g were obtained and immediately immersed in ice-cold buffer solution. The experimental protocol was approved by the Human Study Committee of the East Orange Veterans Administration Hospital and the College of Medicine and Dentistry of New Jersey, New Jersey Medical School. Informed consent was obtained prior to surgery.

Animals

Male rats of the Wistar strain weighing 200–250 g were used. They were fed Purina rat chow prior to the experiments. In certain experiments, animals were treated with phenobarbital (100 mg/kg body wt per day, i.p.) for 5 days. This drug produces proliferation of the smooth endoplasmic reticulum of the liver, thereby increasing the activity of "drug-metabolizing" (hydroxylating) enzymes in the microsomal fraction (9).

Preparation of unlabeled compounds

5β-Cholestane-3α,7α,12α,25-tetrol (mp 189–191°C) was prepared from cholic acid and purified as described by Dayal, et al. (10). 5β-Cholestane-3α,7α,12α,24α,25-pentol (mp 212–214°C), 5β-cholestane-3α,7α,12α,24β,25-pentol (mp 203–205°C) and 5β-cholestane-3α,7α,12α,25,26-pentol were synthesized from 5β-cholestane-3α,7α,12α,25-tetrol as described previously (6, 10, 11). 5β-Cholestane-3α,7α,12α,23ξ,25-pentol (mp 210–211°C) was isolated from the feces of CTX patients, purified and crystallized as previously described (11).

Preparation of labeled compounds

5β-[G-^{3}H]Cholestane-3α,7α,12α,25-tetrol (5 mg) was prepared by the Wilzbach procedure at New England Nuclear, Boston, MA (12), and purified by column and thin-layer chromatography (2, 10) to constant specific radioactivity (3.66×10^{7} dpm/mg; radiopurity 98.4%).

5β-[26,27-^{14}C]Cholestane-3α,7α,12α,25-tetrol was prepared as follows. To a solution of methyl magnesium iodide prepared from 50 mg of magnesium, 2.4 mg of [^{14}C]methyl iodide (0.25 mCi; New England Nuclear) and 347 mg of unlabeled CH_3I in 5 ml of dry ether, and 60 mg of methyl homocholate (10, 13) in 4 ml of anhydrous benzene were added dropwise with stirring. The mixture was refluxed on a water bath for 1 hr. The ether was distilled off and the residual mixture was refluxed for an additional 2 hr and allowed to stand overnight at room temperature. The reaction mixture was then decomposed with a saturated solution of ammonium chloride at 0°C and extracted with ethyl acetate. After removing the solvent under reduced pressure, the residue was hydrolyzed with 2 ml of methanolic potassium hydroxide (10%; w/v) for 1 hr on a water bath (60°C). The hydrolyzate was poured into a large amount of ice and stirred. The resulting precipitate (35 mg) was purified on a neutral alumina column (Grade IV, Calbiochem, Los Angeles, CA) yielding 28 mg of the pure 5β-[26,27-^{14}C]cholestane-3α,7α,12α,25-tetrol (sp act 1.5×10^{6} dpm/mg; radiopurity 97.8%).

Cofactors

NADP, D-glucose-6-phosphate, and glucose-6 phosphate dehydrogenase were obtained from Calbiochem, La Jolla, CA.

GLC

The 5β-cholestanepentol fraction was analyzed as the TMSi derivatives on 180 cm × 4 mm columns packed with 3% QF-1 on 80–100 mesh Gas Chrom Q; column temperature 230°C (Hewlett-Packard, Palo Alto, CA, model 7610 gas chromatograph). The retention times relative to 5β-cholestane (2.95 min.) were: 5β-cholestane-3α,7α,12α,23ξ,25-pentol, 3.94; 5β-cholestane-3α,7α,12α,24α,25-pentol, 4.23; 5β-cholestane-3α,7α,12α,24β,25-pentol, 4.35; 5β-cholestane-3α,7α,12α,25,26-pentol, 4.63 (11).

Mass spectra

Mass spectra of the bile alcohols were obtained with a Varian MAT-111 gas chromatograph-mass spectrometer, Varian Assoc., Palo Alto, CA, as described previously (11).

METHODS

Fractionation of liver homogenates

Male rats were killed by cervical dislocation; their livers were removed immediately and chilled on ice. All subsequent operations were carried out at 0–4°C. The liver was extruded through a tissue press, Harvard Apparatus Co., Millis, MA. A 2 g aliquot was homogenized in a loose-fitting Potter-Elvehjem homogenizer with 8 ml of 0.1 M Tris-Cl buffer pH 7.4 containing 2.5 mM EDTA. The microsomal fraction was obtained by centrifuging the homogenate for 12 min at 20,000 *g*, followed by centrifugation at 100,000 *g* for 1 hr (14). The microsomal pellet was

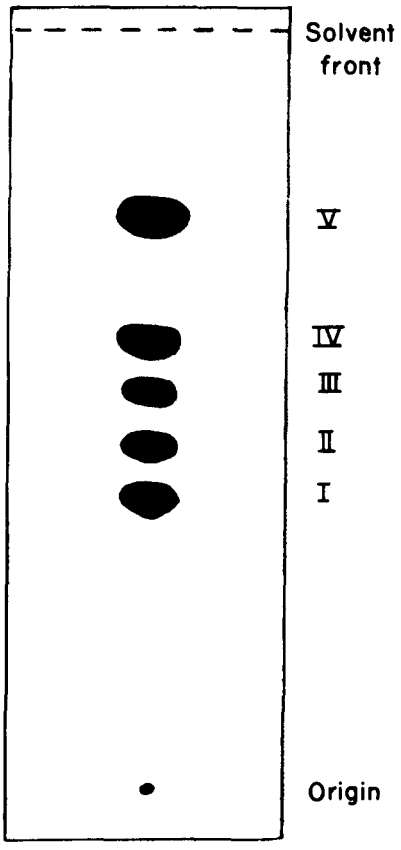

Fig. 1. Thin-layer chromatogram of the 5β-cholestanepentols obtained from an incubation of 5β-[26,27-^{14}C]cholestane-3α,7α,12α,25-tetrol with rat liver microsomes. Samples were applied on 0.25 mm-thick alumina G (Analtech) plates and developed with benzene–ethyl acetate–methanol 75:20:25 (v/v/v). R_f values of the reference compounds used were: I, 5β-cholestane-3α,7α,12α,25,26-pentol, 0.39; II, 5β-cholestane-3α,7α,12α,24α,25-pentol, 0.45; III, 5β-cholestane-3α,7α,12α,24β,25-pentol, 0.52; IV, 5β-cholestane-3α,7α,12α,23ξ,25-pentol, 0.58; V, 5β-cholestane-3α,7α,12α,25-tetrol, 0.75.

washed by resuspension in 0.1 M Tris-Cl buffer pH 7.4 with EDTA, followed by centrifugation for 1 hr at 100,000 *g*. The final pellet was suspended in 0.1 M Tris-Cl buffer pH 7.4 with EDTA in a volume corresponding to the original 20,000 *g* supernatant solution from which it had been prepared.

Protein was determined according to Lowry, et al. (15). The protein content of the microsomal fraction was about 4 mg/ml. In the human studies, the liver tissue obtained was placed immediately in ice-cold 0.1 M Tris-Cl buffer pH 7.4 containing 2.5 mM EDTA and was transported to the laboratory in an ice bath within 1 hr. The tissue fractionation procedures for human and rat liver were identical.

Standard enzyme assays and analysis of incubation mixtures

Labeled substrate, either 5β-[26,27-^{14}C]cholestane-3α,7α,12α,25-tetrol (200 nmoles, sp act 6.55 × 10^2 dpm/nmole) or 5β-[G-^{3}H]cholestane-3α,7α,12α,25-tetrol (200 nmoles, sp act 1.60 × 10^4 dpm/nmole) in methanol, was mixed with 0.15 mg of Tween-80, Fisher Scientific Co., Springfield, N. J. The organic solvent was evaporated away and the dry residue was solubilized by vigorous mixing (Vortex) in 0.1 ml of 0.1 M Tris-Cl buffer pH 7.4. The incubation mixture contained 0.2 mM labeled 5β-cholestane-3α,7α,12α,25-tetrol, 85 mM of Tris-Cl buffer pH 7.4, 1.0 mM EDTA, 1.0 mM NADP, 3.6 mM glucose-6-phosphate, and 7 enzyme units of glucose-6-phosphate dehydrogenase. In the standard assay system (final volume 1.0 ml) the microsomal protein content was about 0.65 mg for the human assay and 0.9 mg for the rat assay. All enzyme assays were carried out in duplicate and zero time controls were run with each experiment. The NADPH-generating system was preincubated for 5 min at 37°C. The incubation of the entire system was carried out with shaking for 10 min at 37°C in air and was terminated by the addition of 1 ml of 10% KOH in methanol–H_2O 90:10 (v/v).

The bile alcohols were extracted with 2 × 2 ml of ethyl acetate, the organic phase was washed twice with water and evaporated to dryness. The 5β-cholestanepentols formed during the incubation were identified and quantitated by a combination of TLC and GLC as follows. The bile alcohols were separated by TLC on 0.25 mm-thick alumina plates (Analtech, Inc., Newark, DE) with benzene–ethyl acetate–methanol 75:20:25 (v/v/v). Activation of the TLC plate at 105°C for 15 min was required and samples were applied when the plate had cooled to 40°C on a constant temperature hot plate. Unlabeled 5β-cholestanepentols (20 μg each) were applied with the extracts as markers. The pertinent spots were made visible with spray reagent consisting of 3.5% phosphomolybdic acid in isopropanol (**Fig. 1**). Enzyme activities were calculated after removing individual pentol spots from the plate and measuring their radioactivity in a liquid scintillation counter (Beckman LS-200 B, Beckman Instruments, Fullerton, CA). Since the specific radioactivity of the substrate was known, the radioactivity data could be expressed in terms of nmoles of products formed.

RESULTS

Conversion of 5β-[G-^{3}H] or 5β-[26,27-^{14}C]-cholestane-3α,7α,12α,25-tetrol to 5β-cholestanepentols

Table 1 illustrates the conversion of 5β-cholestane-3α,7α,12α,25-tetrol into 5β-cholestane-3α,7α,12α,24β,25-pentol and other 5β-cholestanepentols by

TABLE 1. Conversion of labeled 5β-cholestane-3α,7α,12α,25-tetrol to 5β-cholestanepentols by hepatic microsomes[a]

Species	Substrate	Rate of 5β-Cholestanepentol Formation			
		3α,7α,12α, 24β,25-Pentol	3α,7α,12α, 24α,25-Pentol	3α,7α,12α, 23ξ,25-Pentol	3α,7α,12α, 25,26-Pentol[c]
		nmoles/mg protein/10 min			
Man (4)[b]	5β-[G-^{3}H]Cholestane-3α,7α,12α,25-tetrol	0.636 ± 0.042	0.060 ± 0.011	0.294 ± 0.024	0.318 ± 0.020
Rat (5)[b]	5β-[G-^{3}H]Cholestane-3α,7α,12α,25-tetrol	3.380 ± 0.093	3.460 ± 0.145	1.760 ± 0.109	1.260 ± 0.094
	5β-[26,27-^{14}C]Cholestane-3α,7α,12α,25-tetrol	3.240 ± 0.121	3.620 ± 0.157	1.760 ± 0.078	1.630 ± 0.113

[a] Microsomal fractions were prepared and products were analyzed as described in the Methods section. Standard assay conditions were employed.
[b] Number of experiments. Aliquots of the same microsomal preparations from five different rats were used to compare ^{3}H- and ^{14}C-labeled substrates. The data in the table represent the average for each group ± SEM.
[c] 5β-Cholestane-3α,7α,12α,25,26-pentol was most probably of microsomal origin since the contamination of the microsomal fraction with mitochondria was less than 1%. This contamination was estimated by determination of succinate–cytochrome C reductase activity in the microsomal fraction (24).

hepatic microsomal fractions of man and rat. In man the major product (approximately 50% of total pentols) was 5β-cholestane-3α,7α,12α,24β,25-pentol, while in the rat similar amounts of the 24α- and the 24β-epimers were produced. The rates of 5β-cholestanepentol formation were considerably higher in rat than in human liver microsomes.

Identification and radioactive purity of reaction products

To establish the identity and radioactive purity of the 5β-cholestane-3α,7α,12α,24β,25-pentol and the other 5β-cholestanepentols formed from 5β-[26,27-^{14}C]cholestane-3α,7α,12α,25-tetrol, the assay system described in Methods, using rat liver microsomes was scaled up 6-fold. The labeled products formed during a 1 hr incubation were extracted and subjected to thin-layer chromatography on alumina, as described above, without addition of carrier. The bands corresponding to known pentol reference compounds were eluted with methanol and analyzed by GLC–MS and liquid scintillation counting. Known aliquots from each band were then rechromatographed on silica gel G plates, 0.25 mm thick (Analtech) with chloroform–acetone–methanol 35:25:7.5 (v/v/v) (11), eluted with methanol, and analyzed again by GLC–MS and liquid scintillation counting. The purity of a compound was considered established whenever: *1*) its specific radioactivity was constant, *2*) it exhibited a single peak upon GLC, and *3*) its mass spectrum was identical with that of the pure reference compound. These data are summarized in **Table 2**. Such measurements were carried out whenever a group of animals was treated differently from the control group (phenobarbital, starvation) or whenever incubations were carried out under conditions differing from those of the standard assay.

To establish the identity and radioactive purity of the 5β-cholestane-3α,7α,12α,24β,25-pentol formed from 5β-[G-^{3}H]cholestane-3α,7α,12α,25-tetrol by human liver microsomes, the assay system was scaled up about 10-fold and the labeled 5β-cholestanepentols formed after incubation for 1 hr were extracted and separated by TLC on alumina as described above. The band corresponding to 5β-cholestane-3α,7α,12α,24β,25-pentol (about 5 nmoles) was eluted with methanol and the specific radioactivity, determined by GLC and liquid scintillation counting, amounted to 15,320 dpm/nmole. The labeled product was diluted with 3.0 mg of the unlabeled compound and was chromatographed on an alumina column (8.0 × 1.2

TABLE 2. Radioactive purity of biosynthetic 5β-cholestanepentols obtained from incubation of 5β-[26,27-^{14}C]cholestane-3α, 7α,12α,25-tetrol with rat liver microsomes[a]

Products Formed	Specific Radioactivity after TLC on	
	Alumina G	Silica Gel G
	dpm/nmole	
3α,7α,12α,24β,25-Pentol	615	660
3α,7α,12α,24α,25-Pentol	700	647
3α,7α,12α,23ξ,25-Pentol	635	609
3α,7α,12α,25,26-Pentol	698	616

[a] The biosynthetic 5β-[26,27-^{14}C]cholestanepentols obtained from large scale incubation experiments of 5β-[26,27-^{14}C]cholestane-3α,7α,12α,25-tetrol, specific activity 6.55 × 10^2 dpm/nmole, were separated from the incubation mixture by extraction followed by TLC (Alumina G, Silica gel G) and GLC–MS as described in the Results section. GLC retention times and mass spectra of each biosynthetic pentol listed in the table were identical with those of the pure reference compounds.

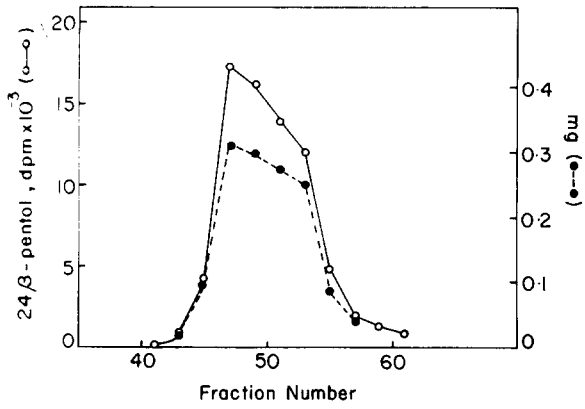

Fig. 2. Column chromatography of biosynthetic 5β-[G-^{3}H]cholestane-3α,7α,12α,24β,25-pentol obtained from a large-scale incubation experiment with human liver microsomes (for details see text). Each column fraction (10 ml) was assayed for ^{3}H by scintillation counting and for mass of pentol by GLC (see Methods section). Elution with increasing proportions of methanol in ethyl acetate. Fractions 1–40, 0–7.5% methanol in ethyl acetate. Fractions 40–60, 10% methanol in ethyl acetate. No radioactivity was detected in fractions 1–40.

cm containing 10 g of neutral alumina, activity Grade V, Bio-Rad AG7, Bio-Rad Laboratories, Richmond, CA). Increasing proportions of methanol in ethyl acetate were used and the product was eluted with 10% methanol in ethyl acetate (11). The specific radioactivity remained constant throughout the 5β-cholestane-3α,7α,12α,24β,25-pentol band, within the precision of measurement estimated as ±7% (**Fig. 2**).

Properties of the microsomal assay system in the rat

a. *Optimal conditions.* 5β-[26,27-^{14}C]Cholestane-3α,7α,12α,25-tetrol was incubated under various conditions with the microsomal fraction; the corresponding labeled 5β-cholestanepentols formed were separated by TLC and their radioactivity was determined as described in the Methods section. The effects of changes in substrate concentration on the reaction rates are shown in **Fig. 3a**. The enzyme systems appeared to be saturated when the concentration of the substrate, 5β-cholestane-3α,7α,12α,25-tetrol, was 200 nmoles/ml (200 μM). The rate of formation of the 5β-cholestane-3α,7α,12α,24β,25-pentol was proportional to incubation time during at least 25 min. On the other hand, the rates of hydroxylation at C-23, C-24α, and C-26 were constant only for the first 10 min. (Fig. 3b). The relationship between reaction rates and enzyme concentration is illustrated in Fig. 3c. Reaction rates were linear with respect to protein concentration up to 1.3 mg with the exception of 5β-cholestane-3α,7α,12α,23ξ,25-pentol. When the microsomal system was adjusted to a pH of 7.7 the reaction rate was optimal for 5β-cholestane-3α,7α,12α,24β,25-pentol formation (Fig. 3d). At this pH the conversion was higher with Tris-Cl buffer than with potassium phosphate buffer. The same optimal pH values (7.7) were observed for the other 5β-cholestanepentols formed.

b. *Effect of phenobarbital and starvation on microsomal hydroxylations of 5β-cholestane-3α,7α,12α,25-tetrol.* The effect of phenobarbital administration on the formation of 5β-cholestanepentols from 5β-[26,27-^{14}C]-cholestane-3α,7α,12α,25-tetrol is shown in **Table 3**. The drug stimulated the formation of all four pentols studied, but to different degrees. The 24α-hydroxylation appeared to be enhanced to a considerably greater extent than the 24β-hydroxylation. Fasting for 48 hr appeared to have no significant effect on any of the hydroxylations (Table 3).

c. *Effect of carbon monoxide on microsomal hydroxylation of 5β-cholestane-3α,7α,12α,25-tetrol.* The rates of hydroxylation of 5β-[26,27-^{14}C]cholestane-3α,7α,12α,25-tetrol by the microsomal fraction were determined in the presence or absence of carbon monoxide as shown in **Table 4**. In the presence of

TABLE 3. Effect of phenobarbital treatment and fasting on hydroxylation of 5β-[26,27-^{14}C]cholestane-3α,7α,12α,25-tetrol by rat liver microsomes[a]

Treatment	Rate of Formation of 5β-Cholestane-			
	3α,7α,12α, 24β,25-Pentol	3α,7α,12α, 24α,25-Pentol	3α,7α,12α, 23ξ,25-Pentol	3α,7α,12α, 25,26-Pentol
	nmoles/mg protein/10 min			
None (5)[b]	3.32 ± 0.124	3.52 ± 0.099	2.16 ± 0.124	1.00 ± 0.078
Phenobarbital[c] (4)[b]	6.14 ± 0.244	19.00 ± 1.125	7.66 ± 0.648	3.78 ± 0.343
Fasting[d] (4)[b]	3.56 ± 0.119	4.44 ± 0.151	2.46 ± 0.331	1.60 ± 0.366

[a] Microsomal fractions were prepared and products were analyzed as described in the Methods section. Standard assay conditions were employed.
[b] Number of rats in each group. The data in the table represent the average for each group ± SEM.
[c] The animals were injected daily for 5 days with 100 mg of phenobarbital/kg body wt, i.p., and were killed 3 hr after the last injection.
[d] The animals were deprived of food for 48 hr. Water was supplied ad lib.

carbon monoxide all hydroxylations were strongly inhibited.

Optimal conditions for the formation of 5β-cholestane-3α,7α,12α,24β,25-pentol in human hepatic microsomes

5β-[G-^{3}H]Cholestane-3α,7α,12α,25-tetrol was incubated under various conditions with the microsomal fraction. The corresponding labeled 5β-cholestane-3α,7α,12α,24β,25-pentol was separated from the other 5β-cholestanepentols formed by TLC and its radioactivity was determined as described in the Methods section. Reaction rates were linear with enzyme concentration up to about 0.75 mg microsomal protein (**Fig. 4a**). The rate of 5β-cholestane-3α,7α,12α,24β,25-pentol formation was constant up to 18 min (Fig. 4b). The effect of changes in substrate concentration on the initial velocity of the reaction is shown in Fig. 4c. The optimal pH was approximately 7.7 (Fig. 4d).

DISCUSSION

The results of the present paper demonstrate that the activity of the hepatic microsomal enzyme converting 5β-cholestane-3α,7α,12α,25-tetrol to 5β-cholestane-3α,7α,12α,24β,25-pentol can be determined by an isotope incorporation procedure. The biosynthetic 5β-cholestane-3α,7α,12α,24β,25-pentol can be well separated from its substrate and from the other 5β-cholestanepentols formed by TLC on alumina (Fig. 1). The sensitivity of the method is such that 0.02 nmole of 5β-cholestane-3α,7α,12α,24β,25-pentol can be detected when [G-^{3}H]5β-cholestane-3α,7α,12α,25-tetrol was used for the conversion (Table 1).

^{14}C-Labeled substrates are usually preferred to randomly tritiated materials (16). However, [G-^{3}H]5β-cholestane-3α,7α,12α,25-tetrol could be prepared with a higher specific radioactivity than the ^{14}C-labeled compound and was therefore used when enzyme activities were relatively low and maximum sensitivity was required for the assay system. In the present case, using rat liver microsomes, we were able to show that the amounts of the 5β-cholestanepentols calculated from the specific activities of either ^{3}H- or ^{14}C-labeled substrates were similar (Table 1). It can be clearly seen that little tritium was lost during the side-chain hydroxylations, except in the case of 5β-cholestane-3α,7α,12α,25,26-pentol, where ^{3}H was lost from a terminal carbon. This observation agrees with the findings of Danielsson and coworkers (17, 18) that the tritium is located mainly in the terminal carbons of the side chain when bile alcohols are tritiated according to the technique of Wilzbach.

The identity and radioactive purity of the reaction products were demonstrated. In the case of the ^{14}C-labeled 5β-cholestanepentols formed by rat liver microsomes, their specific radioactivities remained constant after TLC purification on alumina G and

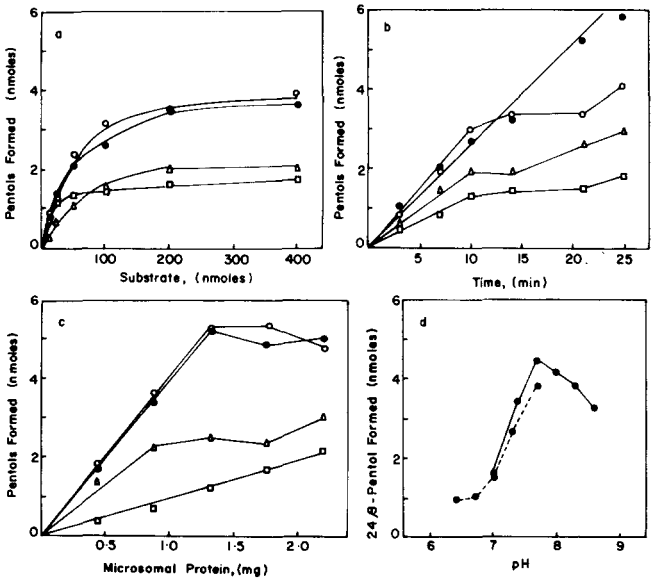

Fig. 3. Effect of substrate concentration (a), time (b), enzyme concentration (c), and pH (d) on hydroxylation of 5β-[26,27-^{14}C]-cholestane-3α,7α,12α,25-tetrol by rat microsomal fraction. Standard assay conditions were employed, except for substrate concentration in (a), incubation time in (b), protein concentration in (c), and pH in (d). In diagram d, the microsomal fraction was suspended in 0.1 M potassium phosphate buffer ●---● or 0.1 M Tris-Cl buffer ●——●. ●——●, 5β-Cholestane-3α,7α,12α,24β,25-pentol; ○——○, 5β-cholestane-3α,7α,12α,24α,25-pentol; △——△, 5β-cholestane-3α,7α,12α,23ξ,25-pentol; and □——□, 5β-cholestane-3α,7α,12α,25,26-pentol.

TABLE 4. Effect of carbon monoxide on hydroxylation of 5β-[26,27-^{14}C]cholestane-3α,7α,12α,25-tetrol by rat liver microsomes[a]

5β-Cholestanepentols Formed	Gas Mixture[b] 4% O_2 96% N_2	Gas Mixture[b] 4% O_2 56% N_2 40% CO	Inhibition
	nmoles/mg protein/ 10 min		%
3α,7α,12α,24β,25-Pentol	2.71	0.751	72.3
3α,7α,12α,24α,25-Pentol	3.21	0.488	84.8
3α,7α,12α,23ξ,25-Pentol	2.15	0.269	87.5
3α,7α,12α,25,26-Pentol	1.50	0.070	95.4

[a] Microsomal fractions were prepared and products were analyzed as described in the Methods section. Standard assay conditions were employed.

[b] The gas mixtures were prepared and analyzed by the Matheson Gas Products Company, East Rutherford, N. J. Gassing was carried out by bubbling the gas mixture through the ice-cold solution for 10 min prior to incubation. The vials were then stoppered and incubated.

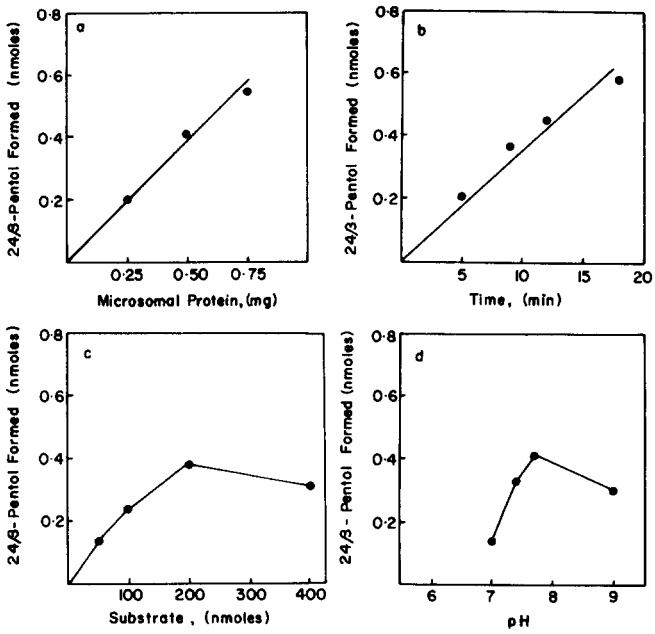

Fig. 4. Effect of enzyme concentration (a), time (b), substrate concentration (c), and pH (d) on hydroxylation of 5β-[G-^{3}H]cholestane-3α,7α,12α,25-tetrol by human liver microsomes. Standard assay conditions were employed, except for protein concentration in (a), incubation time in (b), substrate concentration in (c), and pH in (d).

silica gel G and were identical to the substrate specific activity within the experimental error (±7%) (Table 2). As for the [^{3}H]5β-cholestane-3α,7α,12α,24β,25-pentol formed by human liver microsomes, its specific radioactivity remained constant during TLC and column chromatography (Fig. 2).

Optimal conditions were established for the enzyme assay catalyzing the conversion of 5β-cholestane-3α,7α,12α,25-tetrol to 5β-cholestane-3α,7α,12α,24β,25-pentol and the other 5β-cholestanepentols formed by rat liver microsomes. Using 200 nmoles of substrate, the rate of formation of 5β-cholestane-3α,7α,12α,24β,25-pentol was linear up to 1.3 mg microsomal protein for at least 25 min. In experiments measuring the formation of all four 5β-cholestanepentols, a reaction time of 10 min. and a microsomal protein concentration of 0.9 mg/ml was chosen to assure optimal assay conditions.

Some properties of the 23-, 24-, and 26-hydroxylase systems were studied in the present investigation. Emphasis was placed on experiments that would enable us to make a comparison between these hydroxylase systems and the "drug-metabolizing" enzyme system(s) (19–21). The activity of the latter is increased many times by administration of various drugs, e.g., phenobarbital, and is inhibited by carbon monoxide due to the presence in the system of a CO-sensitive hemoprotein, cytochrome P-450. We, therefore, studied the effect of phenobarbital and carbon monoxide on 23-, 24-, and 26-hydroxylations.

As with the "drug-metabolizing" enzyme system, all hydroxylations were stimulated by the administration of phenobarbital (Table 3) and inhibited by carbon monoxide (Table 4). However, significant differences in the effect of phenobarbital treatment on the hydroxylations at C-23, C-24, and C-26 were observed. The formation of 23-, 24α-, and 26-hydroxylated derivatives of 5β-cholestane-3α,7α,12α,25-tetrol was stimulated three- to fivefold, whereas the increase in the formation of the 24β-derivative was about twofold. These results and the differences in optimal conditions for the hydroxylations at C-23, C-24α, C-24β, and C-26 point to the possibility that the hydroxylase systems are not identical. Similar conclusions were reached by Cronholm and Johansson in their studies of the microsomal hydroxylation of 5β-cholestane-3α,7α,12α-triol (4). In contrast to experiments with 7α-hydroxy-4-cholesten-3-one 12α-hydroxylase, deprivation of food did not affect the rates of hydroxylation of the side chain of 5β-cholestane-3α,7α,12α,25-tetrol. This finding does not support the hypothesis that hydroxylations of C-23, C-24, and C-26 are catalyzed by different enzyme systems.

The effects of time, substrate concentration, enzyme concentration, and pH on the hydroxylation of 5β-cholestane-3α,7α,12α,25-tetrol by human liver microsomes were found to be similar to those of the rat liver enzyme. Since, in man, 5β-cholestane-3α,7α,12α,24β,25-pentol is the major pentol formed (40–50%) and is rapidly converted to cholic acid (6), studies of identity and radioactive purity were confined to this substrate (Fig. 2), and optimal conditions for its assay were demonstrated (Fig. 4). Although it is not known whether this hydroxylase activity undergoes cyclic changes, as does the microsomal cholesterol 7α-hydroxylase (22), in the present study, all liver specimens were obtained at approximately 10 AM in order to minimize the effect of a potential diurnal variation. The sensitivity of the standard assay system described can be increased by using labeled 5β-cholestane-3α,7α,12α,25-tetrol with high specific activity so that small quantities of liver tissue obtained by percutaneous biopsy can be tested.

Recently, cerebrotendinous xanthomatosis (CTX), a disease associated with defective side-chain oxidation in bile acid synthesis, has been described (23). We have previously suggested that a principal biochemical defect in CTX may be associated with a deficiency of an enzyme system catalyzing the conversion of 5β-cholestane-3α,7α,12α,25-tetrol to 5β-cholestane-3α,7α,12α,24β,25-pentol (6). It is hoped

that the enzyme assay of the 24β-hydroxylase described in this paper or analogous assays of other hydroxylases will be useful in estimating relative rates of bile acid synthesis in patients with CTX or related disorders of sterol metabolism. Such assays may well be helpful in defining the inherited enzymatic abnormality and in assessing the efficacy of different treatments in restoring normal hepatic function and reversing clinical problems.

This work was supported by U.S. Public Health Service grants AM 05222, HL 10894, and HL 17818, and by a National Science Foundation grant BMS 75-01168.

Manuscript received 12 May 1976 and accepted 10 August 1976.

REFERENCES

1. Setoguchi, T., G. Salen, G. S. Tint, and E. H. Mosbach. 1974. A biochemical abnormality in cerebrotendinous xanthomatosis: Impairment of bile acid biosynthesis associated with incomplete degradation of the cholesterol side chain. *J. Clin. Invest.* **53:** 1393–1401.
2. Salen, G., S. Shefer, T. Setoguchi, and E. H. Mosbach. 1975. Bile alcohol metabolism in man: Conversion of 5β-cholestane-3α,7α,12α,25-tetrol to cholic acid. *J. Clin. Invest.* **56:** 226–231.
3. Yamada, T. 1966. Stero-bile acids and bile alcohols XCIII. Metabolism of 5β-cholestane-3α,7α,12α,25-tetrol. *Hiroshima J. Med. Sci.* **15:** 375–390.
4. Cronholm, T., and G. Johansson. 1970. Oxidation of 5β-cholestane-3α,7α,12α-triol by rat liver microsomes. *Eur. J. Biochem.* **16:** 373–381.
5. Björkhem, I., J. Gustafsson, G. Johansson, and B. Persson. 1975. Biosynthesis of bile acids in man: Hydroxylation of the C_{27}-steroid side chain. *J. Clin. Invest.* **55:** 478–486.
6. Shefer, S., F. W. Cheng, B. Dayal, S. Hauser, G. S. Tint, G. Salen, and E. H. Mosbach. 1976. A 25-hydroxylation pathway of cholic acid biosynthesis in man and rat. *J. Clin. Invest.* **57:** 897–903.
7. Masui, T., and E. Staple. 1966. The formation of bile acids from cholesterol: The conversion of 5β-cholestane-3α,7α,12α-triol-26-oic acid to cholic acid via 5β-cholestane-3α,7α,12α,24ξ-tetrol-26-oic acid I by rat liver. *J. Biol. Chem.* **241:** 3889–3893.
8. Gustafsson, J. 1975. Biosynthesis of cholic acid in rat liver: 24-Hydroxylation of 3α,7α,12α-trihydroxy-5β-cholestanoic acid. *J. Biol. Chem.* **250:** 8243–8247.
9. Burger, P. C., and P. B. Herdson. 1966. Phenobarbital-induced fine structure changes in rat liver. *Amer. J. Pathol.* **48:** 793–803.
10. Dayal, B., S. Shefer, G. S. Tint, G. Salen, and E. H. Mosbach. 1976. Synthesis of 5β-cholestane-3α,7α,12α,25-tetrol, and 5β-cholestane-3α,7α,12α,24ξ,25-pentol. *J. Lipid Res.* **17:** 74–77.
11. Shefer, S., B. Dayal, G. S. Tint, G. Salen, and E. H. Mosbach. 1975. Identification of pentahydroxy bile alcohols in cerebrotendinous xanthomatosis: Characterization of 5β-cholestane-3α,7α,12α,24ξ,25-pentol and 5β-cholestane-3α,7α,12α,23ξ,25-pentol. *J. Lipid Res.* **16:** 280–286.
12. New England Nuclear Catalog. 1975. New England Nuclear, Boston, Mass. 14–15.
13. Svoboda, J. A., M. J. Thompson, and W. E. Robbins. 1968. 3β-Hydroxy-24-norchol-5-en-23-oic acid—A new inhibitor of the Δ^{24}-sterol reductase enzyme system(s) in the tobacco hornworm, *Manduca sexta* (Johannson). *Steroids.* **12:** 559–570.
14. Danielsson, H., and K. Einarsson. 1966. On the conversion of cholesterol to 7α,12α-dihydroxycholest-4-en-3-one. *J. Biol. Chem.* **241:** 1449–1454.
15. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
16. Panveliwalla, D. K., D. Pertsemilidis, and E. H. Ahrens, Jr. 1974. Tritiated bile acids: problems and recommendations. *J. Lipid Res.* **15:** 530–532.
17. Danielsson, H., A. Kallner, R. J. Bridgwater, T. Briggs, and G. A. D. Haslewood. 1962. On the extent of the conversion of scymnol to cholic acid in the rat. *Acta Chem. Scand.* **16:** 1766–1769.
18. Danielsson, H., and T. Kazuno. 1964. On the formation of cholic acid in the bile fistula rat from some naturally occurring polyhydroxy bile sterols. *Acta Chem. Scand.* **18:** 1157–1163.
19. Hayaishi, O. 1969. Enzymic hydroxylation. *Annu. Rev. Biochem.* **38:** 21–35.
20. Conney, A. H. 1967. Pharmacological implications of microsomal enzyme induction. *Pharmacol. Rev.* **19:** 317–366.
21. Orrenius, S. 1968. *In* Symposium on the Interaction of Drugs and Subcellular Components in Animal Cells. P. N. Campbell, editor. Churchill, London. 97.
22. Danielsson, H. 1972. Relationship between diurnal variations in biosynthesis of cholesterol and bile acids. *Steroids.* **20:** 63–72.
23. Salen, G., and S. M. Grundy. 1973. The metabolism of cholestanol, cholesterol, and bile acids in cerebrotendinous xanthomatosis. *J. Clin. Invest.* **52:** 2822–2835.
24. Kuff, E. L., and W. C. Schneider. 1954. Intracellular distribution of enzymes. XII. Biochemical heterogeneity of mitochondria. *J. Biol. Chem.* **206:** 677–685.