Studies on the 12α and 26-Hydroxylation of Bile Alcohols by Rabbit Liver Microsomes

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ABSTRACT

12α-Hydroxylation of two C_{27}-steroids by rabbit liver microsomes was studied. Optimal assay conditions were determined with 7α-hydroxy-4-cholesten-3-one and 5β-cholestane-3α,7α-diol as substrates. The rate of 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one was found to be greater than that of 5β-cholestane-3α,7α-diol by ca. 60%. Microsomal 26-hydroxylation of 5β-cholestane-3α7α-diol was also measured, and the ratio of 26-hydroxylation to 12α-hydroxylation of 5β-cholestane-3α,7α-diol was found to be ca. 0.4. Rabbit liver 12-αhydroxylase was more active than that of three other species (man, rat, monkey), explaining in part the predominance of cholic acid in rabbit bile.

INTRODUCTION

The mechanism whereby cholesterol is converted into bile acids in vertebrates has been studied extensively in recent years (1). The initial steps in the formation of the main primary bile acids, cholic acid and chenodeoxycholic acid, are probably identical: either 5β-cholestane-3α,7α-diol (I) or 7α-hydroxy-4-cholesten-3-one (II) is the last precursor common to both of the primary bile acids. At present it is not known with certainty whether the diol (I) or the unsaturated ketone (II) is the preferred substrate in the formation of cholic acid (2,3). Presumably 26–hydroxylation of I and II would yield predominantly chenodeoxycholic acid, whereas 12α-hydroxylation, followed by 26-hydroxylation, would result in the formation of cholic acid. It is currently assumed that the relative activities of the 12α- and 26-hydroxylases determine the cholic acid to chenodeoxycholic acid ratio in biliary bile acids (3,4).

Rabbit bile has an unusually high ratio of cholic acid (plus its bacterial metabolite, deoxycholic acid) to chenodeoxycholic acid, compared to that of most other mammalian species (5). It seemed of interest therefore to determine relative rates of 12α- and 26-hydroxylations of 7α-hydroxy-4-cholesten-3-one and 5β-cholestane-3α,7α-diol by rabbit liver microsomes to find out whether biliary bile acid composition reflects the enzyme activities.

MATERIALS AND METHODS

Labeled Substrates

[^{3}H]-5β-Cholestane-3α,7α-diol was synthesized according to Björkhem et al. (6) from [^{3}H]-chenodeoxycholic acid by electrolytic coupling with isovaleric acid, and purified by column chromatography on aluminum oxide grade III (Bio-Rad Labs, Richmond, CA). Elution with increasing concentrations of ethyl acetate-benzene yielded white crystals, mp 80 C, sp act 0.8 μCi/mg, radioactive purity 97% (Checked by thin layer chromatography [TLC] on 0.25 mm alumina G plates [Analtech Wilmington, DE], solvent system: chloroform: acetone:methanol 7:5:0.3 v/v/v).

[^{3}H]-7α-Hydroxy-4-cholesten-3-one was prepared as described by Björkhem et al. (6): [^{3}H]-5β-Cholestane-3α,7α-diol obtained as above was oxidized with aluminum-tertbutoxide; the resulting [^{3}H]-7α-hydroxy-5β-cholestane-3-one was purified by column chromatography on aluminum oxide grade III and dehydrogenated with SeO_2 in ethanol. The crude reaction product was purified by column chromatography on aluminum oxide grade IV and eluted with increasing concentrations of ethyl acetate in benzene; crystals, mp 181 C, sp act 0.75 μCi/mg, radioactive purity 98% (TLC on 0.25 mm plates of Silica Gel G [Analtech, Wilmington, DE], solvent system: benzene:ethyl acetate 6:4 v/v).

Unlabeled Reference Compounds

5β-Cholestane-3α,7α-diol and 7α-hydroxy-4-cholesten-3-one were synthesized in the same manner as the labeled substrates.

5β-Cholestane-3α,7α,12α-triol was prepared by electrolytic coupling of cholic acid and isovaleric acid, as described by Bergström and Krabisch (7), mp 184-185 C.

7α,12α-Dihydroxy-4-cholesten-3-one was

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synthesized as described by Berséus et al. (8), mp 228-229 C.

5β-Cholestane-3α,7α,26-triol was synthesized from chenodeoxycholic acid by electrolytic coupling with methyl-2-methyl-3-carboxypropionate, by the method described by Berséus and Danielsson (9,10), with modifications as follows: Methyl-2-methyl-3-carboxypropionate (3 g) was prepared by refluxing 5 g methyl succinic anhydride (K & K Labs, Cleveland, OH) with 3 ml anhydrous methanol for 1.5 hr; the product was reacted with 500 mg of chenodeoxycholic acid in an electrolytic bath surrounded with ice, containing 200 ml anhydrous methanol, and 25 ml of 0.5 N sodium methoxide: methanol (Applied Science Labs, State College, PA). Platinum electrodes (2 cm diameter) were placed at 0.3 cm distance, and the electrolsis was run 2 hr at ca. 1-1.5 Amp. The reaction mixture was diluted with an equal amount of water and extracted twice with 100 ml ether. The ether extracts were washed twice with 50 ml 5% Na_2CO_3 to dissolve the unreacted free acids, washed with water, dried over anhydrous Na_2SO_4, and evaporated. The crude methyl 3α,7α-dihydroxy-5β-cholestan-26-oate was purified by preparative TLC on Silica Gel G plates, 500 μ thick (Analtech), using known reference compounds as standards. The plates were developed in a solvent system containing chloroform:acetone:methanol (7:5:0.3 v/v/v), and narrow bands along the two edges were sprayed with 3.5% phosphomolybdic acid in isopropanol. The band with the same R_f as the reference compound was scraped off the plates and extracted by stirring 15 min with 100 ml acetone. Evaporation of the solvent and crystallization from acetone: water yielded 60 mg methyl 3α,7α-dihydroxy-5β-cholestan-26-oate (purity by TLC > 99% [mp 50-55 C]). Purified ester (50 mg) was reduced with 50 mg $LiAlH_4$ (Alpha Inorganics, Beverly, MA) in 20 ml anhydrous ether by refluxing the reaction mixture for 2 hr. After decomposition of the unreacted $LiAlH_4$ with ethyl acetate, the reaction mixture was acidified with 2N HCl, the organic layer was separated, washed with water, dried over anhydrous Na_2SO_4 and evaporated. The crude reaction product was purified by preparative TLC, using the same type of plates and solvent system as for the purification of the ester, with pure 5β-cholestane-3α,7α,26-triol and methyl 3α,7α-dihydroxy-5β-cholestan-26-oate applied as external standards on both sides of the plates. The area corresponding to the R_f values of the reference compounds was scraped from the plates and extracted with acetone. Evaporation of the solvent and crystallization of the compounds from acetone: water (1:1 v/v) yielded 12 mg unreacted ester and 18 mg pure 5β-cholestane-3α,7α,26-triol, mp 149-151 C, mass spectrum identical with that published by Björkhem and Gustafsson (11) with main peaks at m/e 546 (M-90), 531 [M-(90+15)], 456 [M-(2x90)], 441 [M-(2x90+15)], 366 [M-3x90)], and 351 [M-(3x90+15)].

It is of practical interest that the purification of most of the intermediate reaction products, as well as that of the final bile alcohols, could be performed by preparative TLC on activated alumina plates, rather than silica gel, as described in the experimental section for the synthesis of 5β-cholestane-3α,7α,26-triol. This procedure avoids time-consuming purification procedures by column chromatography.

Animals and Human Subjects

The rabbits used were male New Zealand whites weighing ca. 1 kg. Rats were males of the Sprague-Dawley strain (Charles River Breeding Laboratories, Wilmington, MA) weighing between 200-250 g. The Rhesus monkeys used were 2 males and 2 females which weighed between 2.7-3.3 kg. All animals were given food and water ad libitum prior to sacrifice. The livers of the animals were always removed between 10 and 11 a.m. to minimize possible effects of diurnal variations. Liver biopsies from the human subjects were kindly provided by G. Salen (12).

Preparation of Microsomes and Assay Conditions

Male rabbits weighing ca. 1 kg were used. Liver homogenates (30% w/v) were prepared and used for preparation of microsomal suspensions of ca. 30% concentration, as described for the preparation of rat liver microsomes (13). Protein concentration was determined by the method of Lowry et al. (14). Standard incubations were performed at 37 C for 20 min; 100 nmol of the substrate dissolved in 50 μl acetone were preincubated for solubilization for 10 min at room temperature with 0.3 ml of a solution containing 0.6 mg bovine serum albumin (Calbiochem, Monsey, NY). The standard assay system for all three microsomal enzymes contained, in a vol of 2.2 ml: 0.3ml of solubilized substrate (100 nmol substrate), 0.5 ml of the 30% microsomal suspension, 1.0 ml of a reduced nicotinamide adenine dinucleotide phosphate generating system (70 mM potassium phosphate buffer, pH 7.4; 2.25 mM $MgCl_2$; 1.25 mM nicotinamide adenine dinucleotide phosphate; 2.5 mM glucose-6-phosphate and 5 units glucose-6-phosphate dehydrogenase), and 0.4 ml of homogenizing medium (0.3 M sucrose, 75 mM nicotinamide, 2 mM ethylenedia

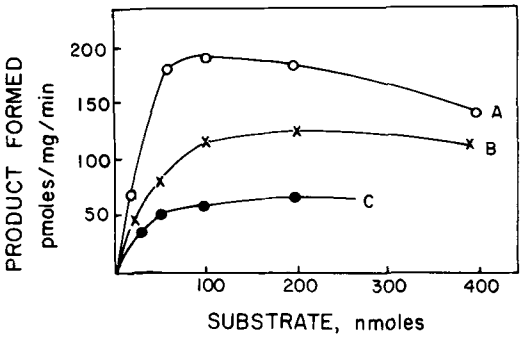

FIG. 1. Effect of substrate concentration 12α- and 26-hydroxylation rates of 5β-cholestane-3α, 7α-diol and 7α-hydroxy-4-cholesten-3-one. Standard assay conditions were used except for substrate concentrations. A = 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one; B = 12α-hydroxylation of 5β-cholestane-3α, 7α-diol; C = 26-hydroxylation of 5β-cholestane-3α,7α-diol. All points represent the average of duplicate determinations.

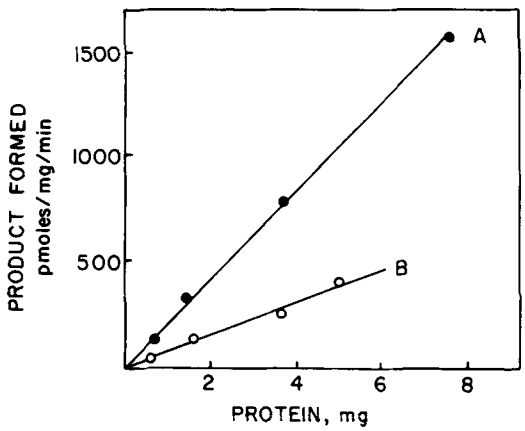

FIG. 2. Effect of microsomal protein concentration on the 12α-hydroxylation of 5β-cholestane-3α,7α-diol and 7α-hydroxy-4-cholesten-3-one. Standard assay conditions (0.6 mg albumin and 100 nmol substrate per tube), except for microsomal protein concentrations. A = 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one; B = 12α-hydroxylation of 5β-cholestane-3α,7α-diol. All points represent the average of duplicate determinations.

minetetraacetic acid, and 20 mM mercaptoethanol).

Incubations were terminated by the addition of 2 ml methanol, and the reaction products were extracted with 20 vols of chloroform: methanol (3:1 v/v). The chloroform extracts were evaporated under nitrogen and the incubation products were separated by TLC on Silica Gel G (Analtech) plates. When [^{3}H]-7α-hydroxy-4-cholesten-3-one was used as substrate, unlabeled 7α-hydroxy-4-cholesten-3-one and 7α,12α-dihydroxy-4-cholesten-3-one were added as carriers and the plates were developed with ethyl acetate:benzene (6:4 v/v). The compounds were made visible by exposing the plates to iodine vapor. Unlabeled carriers for the extracts from the incubation of [^{3}H]-3α,7α-dihydroxy-5β-cholestane used as substrate were 5β-cholestane-3α,7α-diol (R_f 0.90), 5β-cholestane-3α,7α,26-triol (R_f 0.59), and 5β-cholestane-3α,7α,25-triol (R_f 0.64). The solvent system used was chloroform:acetone: methanol (7:5:0.3 v/v/v) on alumina plates, 0.25 mm thick, which were activated by heating 30 min at 100 C. The compounds were made visible by spraying the plates with 3.5% phosphomolybdic acid in isopropanol.

The pertinent spots were scraped from the plates and counted for radioactivity in vials containing 0.3 ml water and 14 ml of scintillation solution (5 g of 2,5-diphenyloxazole and 100 g naphthalene per liter of dioxane). Enzyme activities were expressed in picomoles of product formed per mg protein per min.

RESULTS

Identification of Incubation Products

Incubation products were identified by gas chromatography-mass spectrometry. Retention times and mass spectra of the incubation products 7α-hydroxy-4-cholesten-3-one, 5β-cholestane-3α,7α,12α-triol, and 5β-cholestane-3α,7α-26-triol were identical with those of the authentic reference compounds synthesized as described in the experimental section.

Properties of the Microsomal Assay System

The effects of substrate concentrations on hydroxylation rates are presented in Figure 1 for the following substrates: A = 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one; B = 12α-hydroxylation of 5β-cholestane-3α,7α-diol; C = 26-hydroxylation of 5β-cholestane-3α,7α-diol. In the standard assay systems containing ca. 1-2 mg protein per tube, 100 nmol substrate "solubilized" with 0.6 mg albumin was used in all three hydroxylation reactions studied to ensure that the enzymes were saturated. The relationship between reaction rate and enzyme concentration is illustrated in Figure 2 for the 12α-hydroxylations of the two substrates studied (A = 7α-hydroxy-4-cholesten-3-one; B = 5β-cholestane-3α,7α-diol). Proportionality was observed when the protein concentration ranged up to ca. 8 mg per tube in the standard assay system. The rate of formation of the reaction products was found to be linear with time during a 40 min period, and an incubation time of 20 min was chosen for the standard assay.

The optimum pH for the enzyme activities studied was ca. 7.4. Figure 3 illustrates the relative reaction rates of the hydroxylations studied (A = 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one; B = 12α-hydroxylation of 5β-cholestane-3α,7α-diol; C = 26-hydroxylation of 5β-cholestane-3α,7α-diol), expressed in picomoles reaction products formed per mg microsomal protein per min.

A comparison between 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one catalyzed by rabbit liver microsomes and liver microsomes obtained from other species is illustrated in Figure 4. Liver microsomes of rat, man, and monkey were used for enzyme measurements, and the results indicate that the most active 12α-hydroxylase was that of the rabbit.

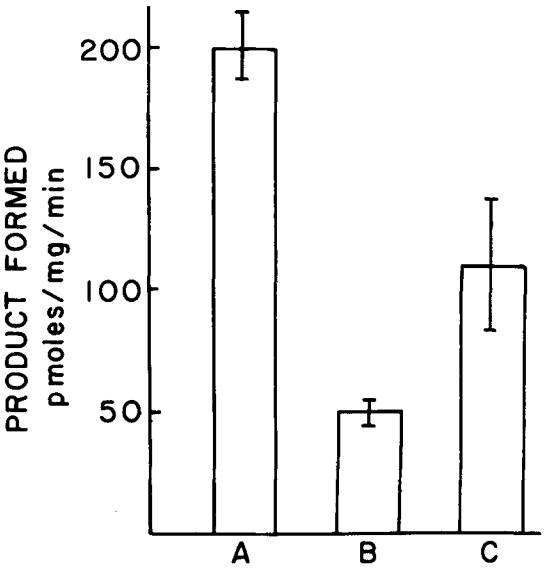

FIG. 3. Relative rates of 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one (A), 26-hydroxylation of 5β-cholestane-3α,7α-diol (B), and 12α-hydroxylation of 5β-cholestane-3α,7α-diol (C), by rabbit liver microsomes. Four determinations were made for each substrate. The vertical lines represent the (±) SEM.

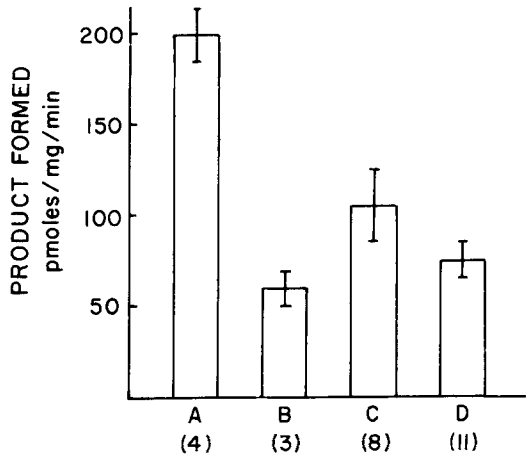

FIG. 4. Species difference in 12α-hydroxylase activity in livers of rabbit (A), man (B), monkey (C), and rat (Sprague-Dawley) (D), determined by using [^{3}H]-7α-hydroxy-4-cholesten-3-one as substrate and the standard assay conditions as described in the experimental section. Enzyme activity is expressed in picomoles 7α,12α-dihydroxy-4-cholesten-3-one formed. The figures in parentheses indicate the number of experiments for each assay. The vertical lines represent the (±) SEM.

DISCUSSION

The rate of 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one by rabbit liver microsomes was found to be ca. 0.2 nmol 12α-hydroxylated product formed per mg protein per min, which is ca. twice as high as the reaction catalyzed by monkey liver and three times higher than that catalyzed by human liver microsomes (Fig. 4). The high 12α-hydroxylase activity is in good agreement with the bile acid composition of rabbit bile, consisting mostly of cholic acid and its bacterial metabolite, deoxycholic acid. A similar correspondence between a high 12α-hydroxylase activity and the predominance of cholic acid in the bile was found in a patient with cerebrotendinous xanthomatosis (15). The 26-hydroxylation of the same substrate by the microsomal fraction of rabbit liver did not occur to a considerable extent under the assay conditions employed.

When one compares the 12α-hydroxylation of the two substrates studied, 7α-hydroxy-4-cholesten-3-one seems to be a more suitable substrate for the microsomal 12α-hydroxylase than 5β-cholestane-3α,7α-diol (Fig. 1). The same figure indicates that 12α-hydroxylation of the diol is much faster than the 26-hydroxylation of the same substrate, but that 26-hydroxylation is nevertheless a noteworthy reaction in the microsomes. This finding is somewhat unexpected inasmuch as according to Björkhem and Gustafsson (11), the ratio of 12α:26-hydroxylation is important in determining the cholic acid:chenodeoxycholic acid ratio in the bile, yet rabbit bile contains practically no chenodeoxycholic acid or its bacterial metabolites. Perhaps in the rabbit, in vivo, the pathway of bile acid synthesis involves 7α-hydroxy-4-cholesten-3-one rather than 5β-cholestane-3α,7α-diol as substrate for the 12α-hydroxylase. This assumption is based on the fact that we found a better correlation between the 12α:26-hydroxylation determined in vitro and

the cholic acid:chenodeoxycholic acid ratio in the bile when 7α-hydroxy-4-cholesten-3-one rather than the diol was used as substrate for the same hydroxylations. These considerations are of course valid only if it is assumed that in vitro measurements of enzyme activity reflect in vivo reaction rates.

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