Comparison of 12α-Hydroxylation of Oxygenated 5α-Cholestanes and Allochenodeoxycholate with Rat Liver Microsomes†

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ABSTRACT: [5α,6α-$^3H_2$]-5α-Cholestane-3α,7α-diol (A), (25*R*)-3α,7α-dihydroxy-[5α,6α-$^3H_2$]-5α-cholestan-26-oic acid (B), (25*R*)-[5α,6α-$^3H_2$]-5α-cholestane-3α,7α,26-triol (C), and [3β-^{3}H]allochenodeoxycholic acid (D) were prepared, characterized, and studied with a rat liver microsomal preparation fortified with 1 m*M* NADPH. The 12α-hydroxylated product formed from each of these substrates was identified by isotopic dilution; the relative reactivity of the four substrates was (A) 100; (B) 87; (C) 135; and (D) 40. The microsomal system showed a requirement for added NADPH and other properties similar to those shown for 12α-hydroxylation of 7α-hydroxycholest-4-en-3-one, which is ultimately converted to cholic acid. Since the coplanar 5α-cholestane-3α,7α-diol is virtually superimposable upon 7α-hydroxycholest-4-en-3-one, and the enzymic requirements are comparable, it is suggested that a single enzyme system may be responsible for 12α-hydroxylation of these substrates.

In earlier reports from this laboratory (Mui et al., 1970, Mui and Elliott, 1971b) allocholic acid was identified as the principal metabolite of allochenodeoxycholic acid in bile from the rat with a cannulated bile duct. This 12α-hydroxylation of the dihydroxy-5α-bile acid stands in marked contrast to the metabolism of the more abundant chenodeoxycholic acid, which is not 12α-hydroxylated in this species but is metabolized in ring B to α- and β-muricholates. Although allocholic acid has been shown to be a major acidic metabolite in the rat of 5α-cholestanol[1] (Karavolas et al., 1965) and of a number of oxygenated 5α-cholestanes [3β,7α-diol (Noll and Elliott, 1969; Noll et al., 1973a; Björkhem and Gustafsson, 1971), 3β,26-diol (Noll et al., 1973b), 3β,7α,26-triol (Noll et al., 1973b; Mui et al., 1970), 3α,7α-diol (Björkhem and Gustafsson, 1971), 3-oxo-7α-ol (Björkhem and Gustafsson, 1971; Björkhem and Einarsson, 1970), 3-oxo-7α,12α-diol (Björkhem and Einarsson, 1970), and 3β,7α-dihydroxy-26-oic acid (Mui and Elliott, 1971a, 1975)], a comparison of data from such studies in the intact animal (Elliott and Hyde, 1971) does not provide a true measure of the ability of the substrate to undergo 12α-hydroxylation because of the various periods of time at which samples were collected from several animals. In order to evaluate the effect of structure of the side chain on optimal activity of 12α-hydroxylation, a series of such derivatives was prepared and studied with a rat liver microsomal preparation as described by Einarsson (1968). Since 5α-cholestane-3β,7α,26-triol is metabolized to a complex spectrum of products by cell-free preparations of rat liver (Mui et al., 1970) during which the 3β-hydroxyl group is isomerized in part to the 3α configuration, derivatives of 5α-cholestane-3α,7α-diol (Björkhem and Gustafsson, 1971) were prepared as more suitable substrates. This report compares the relative yield of 12α-hydroxylated products derived from the above sterol, its 26-hydroxy,26-carboxyl derivatives, and allochenodeoxycholate.

Materials and Methods

[5α,6α-$^3H_2$]-5α-Cholestane-3α,7α-diol (A). A sample of 3β-acetoxycholest-5-en-7-one [mp 163°; lit. mp 157–158° (Wintersteiner and Moore, 1943)] was reduced with 5 Ci of tritium over 5% palladium on carbon (New England Nuclear Corp.) and converted to 7α-acetoxy-[5α,6α-$^3H_2$]-5α-cholestan-3-one (Noll et al., 1973b). After catalytic reduction the C-3 isomers were separated by preparative layer chromatography (PLC) (system 2), characterized by thin-layer chromatography (TLC) and gas–liquid chromatography (GLC), and the appropriate isomer was hydrolyzed with alkali to provide [5α,6α-$^3H_2$]-5α-cholestane-3α,7α-diol, specific activity 1.89 × 10^8 dpm/μmol; mp 167–168°; lit. mp 159–160° (Björkhem and Gustafsson, 1971); R_f 0.12 (system 2). The mass spectrum showed a molecular ion at *m/e* 404, 386 (M − 18) (base peak above *m/e* 43), and other fragment ions at 371 [*M* − (18 + 15)], 368 [*M* − (2 × 18)], 353 [*M* − (2 × 18 + 15)], and 273, 255, 249, 246, 231, 228, and 213, all characteristic of a disubstituted steroid nucleus (Elliott, 1971).

(25R)-3α,7α-Dihydroxy-[5α,6α-$^3H_2$]-5α-Cholestanoic Acid (B). A sample of (25*R*)-[5α,6α-$^3H_2$]-5α-cholestane-3β,7α,26-triol (Noll et al., 1973b) was converted to (25*R*)-3α,7α-dihydroxy-[5α,6α-$^3H_2$]cholestanoic acid (specific activity 1.89 × 10^8 dpm/μmol) via the procedure used for the nonradioactive acid (Mui et al., 1974), and the two acids were found to be chemically identical. The mass spectrum of the tritiated product exhibited fragment ions at *m/e* 416 (*M* − 18) and 398 [*M* − (2 × 18)] with relatively high

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[1] Systematic names of compounds referred to in the text by their trivial names are as follows: chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanic acid; α-muricholic acid, 3α,6β,7α-trihydroxy-5β-cholanic acid; β-muricholic acid, 3α,6β,7β-trihydroxy-5β-cholanic acid; 5α-cholestanol, 5α-cholestan-3β-ol; allochenodeoxycholic acid, 3α,7α-dihydroxy-5α-cholanic acid; allocholic acid, 3α,7α,12α-trihydroxy-5α-cholanic acid; all 5α-cholestanoic acids and cholanic acids are 5α-cholestan-26-oic acids and cholan-24-oic acids.

intensity; additional ions at 273 [M − (18 + side chain)], 255, 246, 231, 228, and 213 are characteristic of a disubstituted steroid nucleus (Elliott, 1971).

(25R)-[5α,6α-$^3H_2$]-5α-Cholestane-3α,7α,26-triol (C). The above C-26 acid was esterified with diazomethane and the ester was reduced under reflux with $LiAlH_4$ in tetrahydrofuran in the usual manner. After purification by partition chromatography and TLC in system 4, the product was crystallized from hot methanol as long needles, mp 156–157°; R_f 0.18 (system 3); 0.55 (system 4), and 0.76 (system 5); RRT[2] on QF-1, 1.89. The mass spectrum showed a molecular ion, *m/e* 420, an ion at 402 (M − 18) (base peak above *m/e* 43), and intense ions at *m/e* 387 [M − (18 + 15)], 384 [M − (2 × 18)], 291 [M − side chain], and other fragment ions at 273, 264, 255, 246, 231, 228, 213, 173, and 159 characteristic for a disubstituted steroid nucleus, and quite similar overall to that reported for 5α-cholestane-3β,7α,26-triol (Noll et al., 1973b). The specific activity was the same as that of the acid.

[3β-^3H]Allochenodeoxycholic Acid (D). This sample was prepared in the manner reported (Mui and Elliott, 1971b); specific activity 1.16 × 10^8 dpm/μmol.

Chromatography and Other Methods. Bile acids and sterols were purified by acetic acid partition chromatography (Matschiner et al., 1957). In this system the fractions have been designated according to the percentage of benzene in hexane; e.g., fraction 60-2 represents the second fraction of the eluent containing 60% benzene in hexane. The substrates used are eluted in the following fractions: 5α-cholestane-3α,7α-diol (A), 0-1; 3α,7α-dihydroxy-5α-cholestanoic acid (B), 0-3, 0-4, and 20-1 (Mui et al., 1974); 5α-cholestane-3α,7α,26-triol (C), 20-1 and 20-2; allochenodeoxycholic acid (D), 20-4, 40-1, 40-2, and 40-3 (Mui and Elliott, 1971b). The 12α-hydroxy derivatives are eluted as follows: 5α-cholestane-3α,7α,12α-triol, 0-3 and 0-4; 3α,7α,12α-trihydroxy-5α-cholestanoic acid, 40-3, 40-4, and 60-1 (Kamat and Elliott, 1972); 5α-cholestane-3α,7α,12α,26-tetrol, 40-4, 60-1, and 60-2 (Kamat and Elliott, 1972); allocholic acid, 60-4, 80-1, and 80-2 (Mui and Elliott, 1971b). Analytical TLC or PLC of bile acids and sterols was carried out as reported (Noll et al., 1973a; Kamat and Elliott, 1972) with the following solvent systems (v/v): system 1, chloroform–methanol–acetic acid, 80:12:3 (Siegfried and Elliott, 1968); system 2, acetone–benzene, 15:85; system 3, acetone–benzene, 3:7; system 4, acetone–benzene, 1:1; system 5, acetone–benzene, 7:3. GLC and mass spectrometry were carried out as reported (Elliott et al., 1969; Noll et al., 1973b). Radioactivity was determined in Bray's solution (Noll et al., 1973b).

Incubations. Male rats of the St. Louis University colony weighing 200–300 g were used. Homogenates of liver were prepared according to Danielsson and Einarsson (1966). Pieces of chilled liver were suspended in four volumes of modified Bucher's medium (Bergström and Gloor, 1955) (0.1 M phosphate, pH 7.4), homogenized in a Potter-Elvehjem (Thomas) homogenizer equipped with a loosely fitting Teflon pestle driven by a motor at medium speed and rotated up and down four times. The homogenate was centrifuged at 800*g* for 10 min and the supernatant was then centrifuged at 20,000*g* for 10 min. Mitochondria were sedimented at 85,000*g* for 10 min from the resuspended 20,000*g* pellet, washed once, and suspended to a concentration of 4 g wet liver weight/ml. The 20,000*g* supernatant fluid was centrifuged at 100,000*g* for 2 hr. The microsomal pellet was resuspended in a volume corresponding to the original 20,000*g* supernatant fluid and centrifuged at 800*g* for 10 min to provide washed microsomes. All incubations were carried out at 37° with mechanical shaking in a Dubnoff shaker bath for specified periods of time. The substrate was added in 50 μl of methanol. With each series of incubations a heated control was included; the subcellular fraction was heated at 100° on a steam bath for 15 min prior to addition of the substrate and/or cofactors. The reaction was terminated by addition of 10 volumes of 95% ethanol. The protein was removed by filtration, the filtrate was evaporated in vacuo, and the residue was taken up in ethanol; recovery of administered radioactivity by this method was 90–95%.

[2] Abbreviation used is: RRT, relative retention time related to methyl deoxycholate as 1.00.

Table I: Some Properties of Rat Liver 12α-Hydroxylase.[a]

Conditions	Relative Activity
Complete system (CS)[b]	100
Mitochondria[b]	5
Boiled microsome + NADPH	0
100,000*g* cytosol[b]	0
CS-NADPH, + NADH	8
CS + Mg^{2+} (5 mM)	110
CS + EDTA (1 mM)	100
CS + allocholic acid (10 nmol)	98
CS + 5α-cholestane-3α,7α,12α-triol (10 nmol)	90

[a] Properties of 5α-steroid 12α-hydroxylase as measured by the formation of 5α-cholestane-3α,7α,12α-triol from 5α-cholestane-3α,7α-diol. [b] Complete system (CS) contained 3.0 ml; microsomal protein, 7.2 mg/ml in 0.1 M phosphate buffer (pH 7.4) with 3 μmol of NADPH and 100 nmol of 5α-cholestane-3α,7α-diol. Mitochondrial and cytosol fractions contained 3.8 and 13.5 mg of protein/ml, respectively. Incubation was carried out at 37° for 10 min.

The products of incubation were monitored by radioassay after TLC in solvent systems 1, 4, or 5; each product was cochromatographed with the authentic compound. For final identification the radioactive samples were separated by acetic acid partition chromatography, mixed with authentic material, and crystallized to constant specific activity.

The progression of hydroxylation measured at 1, 3, 5, 10, and 20 min was determined from a single incubation and from a series of incubations performed with half-quantities of each component. Duplicate sets of incubations were performed with two separate microsomal preparations; results are expressed as the mean of the two sets of data. The following materials were obtained from Sigma Chemical Co., St. Louis: NADPH, EDTA, ATP, NAD^+, NADH, glutathione, and bovine serum albumin.

Results

Microsomal 12α-Hydroxylase. [5α,6α-$^3H_2$]-5α-Cholestane-3α,7α-diol (A) (specific activity 1.89 × 10^8 dpm/μmol) was chosen as substrate to study some of the requirements of microsomal 12α-hydroxylase; the expected product, 5α-cholestane-3α,7α,12α-triol (R_f 0.41), was readily separated from the substrate (R_f 0.81) in solvent system 4 and was monitored by radioassay. Table I shows the results obtained after incubation at pH 7.4 in 0.1 M phosphate buffer with 3 μmol of NADPH and 100 nmol of 5α-cholestane-3α,7α-diol. The enzymic activity was localized primarily in the microsomal fraction; no significant activity was observed with mitochondrial enzymes, and none with boiled

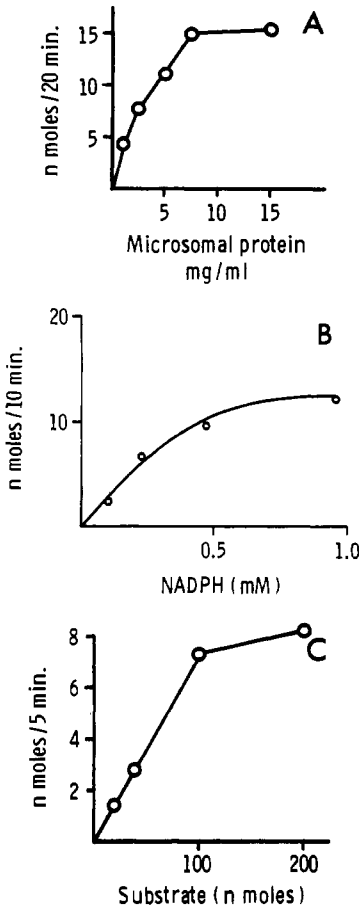

FIGURE 1: Relation of microsomal enzyme to constituents for 12α-hydroxylation of 5α-cholestane-3α,7α-diol. (A) Concentration of protein vs. nanomoles of product formed; (B) concentration of added NADPH vs. nanomoles of product formed; (C) concentration of substrate vs. nanomoles of product formed.

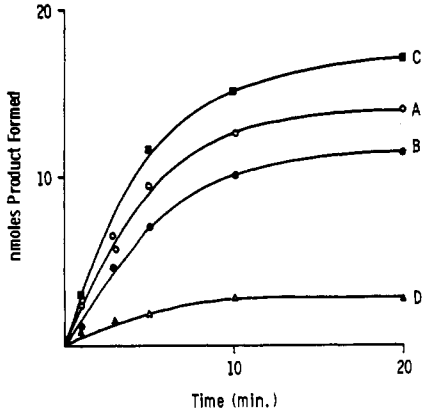

FIGURE 2: Microsomal 12α-hydroxylation of substituted 5α-cholestane-3α,7α-diols and allochenodeoxycholate. The standard assay conditions were used with 100 nmol each of [5α,6α-$^3H_2$]-5α-cholestane-3α,7α-diol (A), (25*R*)-3α,7α-dihydroxy-[5α,6α-$^3H_2$]-5α-cholestanoic acid (B), (25*R*)-[5α,6α-$^3H_2$]-5α-cholestane-3α,7α,26-triol (C), and [3β-^{3}H]allochenodeoxycholic acid (D).

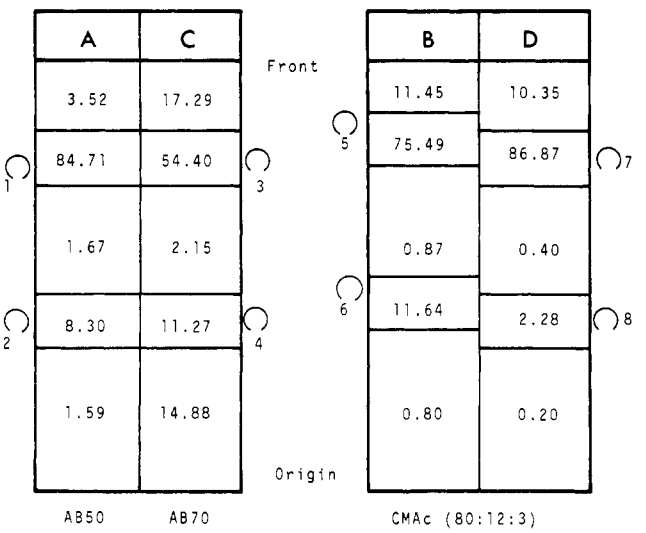

FIGURE 3: Thin-layer chromatography of material from ethanol extracts from incubation of [5α,6α-$^3H_2$]-5α-cholestane-3α,7α-diol (A), (25*R*)-3α,7α-dihydroxy-[5α,6α-$^3H_2$]-5α-cholestanoic acid (B), (25*R*)-[5α,6α-$^3H_2$]-5α-cholestane-3α,7α,26-triol (C), and [3β-^{3}H]allochenodeoxycholic acid (D) with microsomal fraction (system II) for 5 min. The reference compounds are: (1) the nonradioactive diol (A); (2) 5α-cholestane-3α,7α,12α-triol; (3) nonradioactive triol (C); (4) 5α-cholestane-3α,7α,12α-26-tetrol; (5) nonradioactive acid (B); (6) 3α,7α,12α-trihydroxy-5α-cholestanoic acid; (7) allochenodeoxycholic acid; (8) allocholic acid. Solvent systems AB50, AB70, and CMAc are systems 4, 5, and 1, respectively.

microsomes or soluble fraction. NADPH was a necessary cofactor since upon replacement with an equivalent amount of NADH, only 8% of the activity remained. There was no requirement for Mg^{2+}, or other divalent metal ions, since 1 m*M* EDTA had no effect on product formation. Neither 5α-cholestane-3α,7α,12α-triol nor allocholate at a concentration of 10 nmol/3 ml had appreciable effect on formation of product. The activity of 12α-hydroxylase was dependent on the concentration of microsomal protein, NADPH, and the substrate, 5α-cholestane-3α,7α-diol (Figure 1). Accordingly, subsequent studies used a concentration of 7.2–7.5 mg/ml of protein, 1 m*M* NADPH, and 100 nmol of substrate.

Enzymatic Hydroxylation of Substrates. Figure 2 shows the yield of product in nanomoles obtained after incubation of the substrates from 1 to 20 min. Figure 3 shows the results of analysis by TLC of an aliquot (5–20 μl) of the ethanol extract from each of the substrates after incubation for 5 min.

[5α,6α-$^3H_2$]-5α-Cholestane-3α,7α-diol (A). The chromatogram (TLC) of material incubated for 5 min showed 8.3% of the tritium concentrated in the region associated with the product, 5α-cholestene-3α,7α,12α-triol (Figure 3A). After partition chromatography (Figure 4A) two peaks of tritium appeared in fractions 0-1 through 0-4 and in fractions 0-6 through 0-10 (7.0%) corresponding to substrate and product, respectively. The combined material from fractions 0-6 through 0-10 from two incubations was rechromatographed, and half of the residue from fractions 0-3 to 0-5 (1.01×10^6 dpm) was mixed with 37.7 mg of authentic 5α-cholestane-3α,7α,12α-triol [mp 251–252°, lit. mp 251° (Hoshita et al., 1968), 249–251° (Björkhem and Gustafsson, 1971); R_f 0.41 (system 4); RRT on QF-1, 1.74] to give a calculated specific activity of 2.68×10^4 dpm/mg. After three crystallizations the specific activity remained constant (Table II). Thus, 90.7% of the material in this fraction was identified as 5α-cholestane-3α,7α,12α-triol, or 4.84 nmol of product were formed in 5 min.

(25R)-3α,7α-Dihydroxy-[5α,6α-$^3H_2$]-5α-cholestanoic Acid (B). The labeled acid (R_f 0.88, system 1) was converted to more polar products of which 11.6% (Figure 3B) appeared in the region with the mobility of 3α,7α,12α-trihydroxy-5α-cholestanoic acid (R_f 0.53 system 1). After parti-

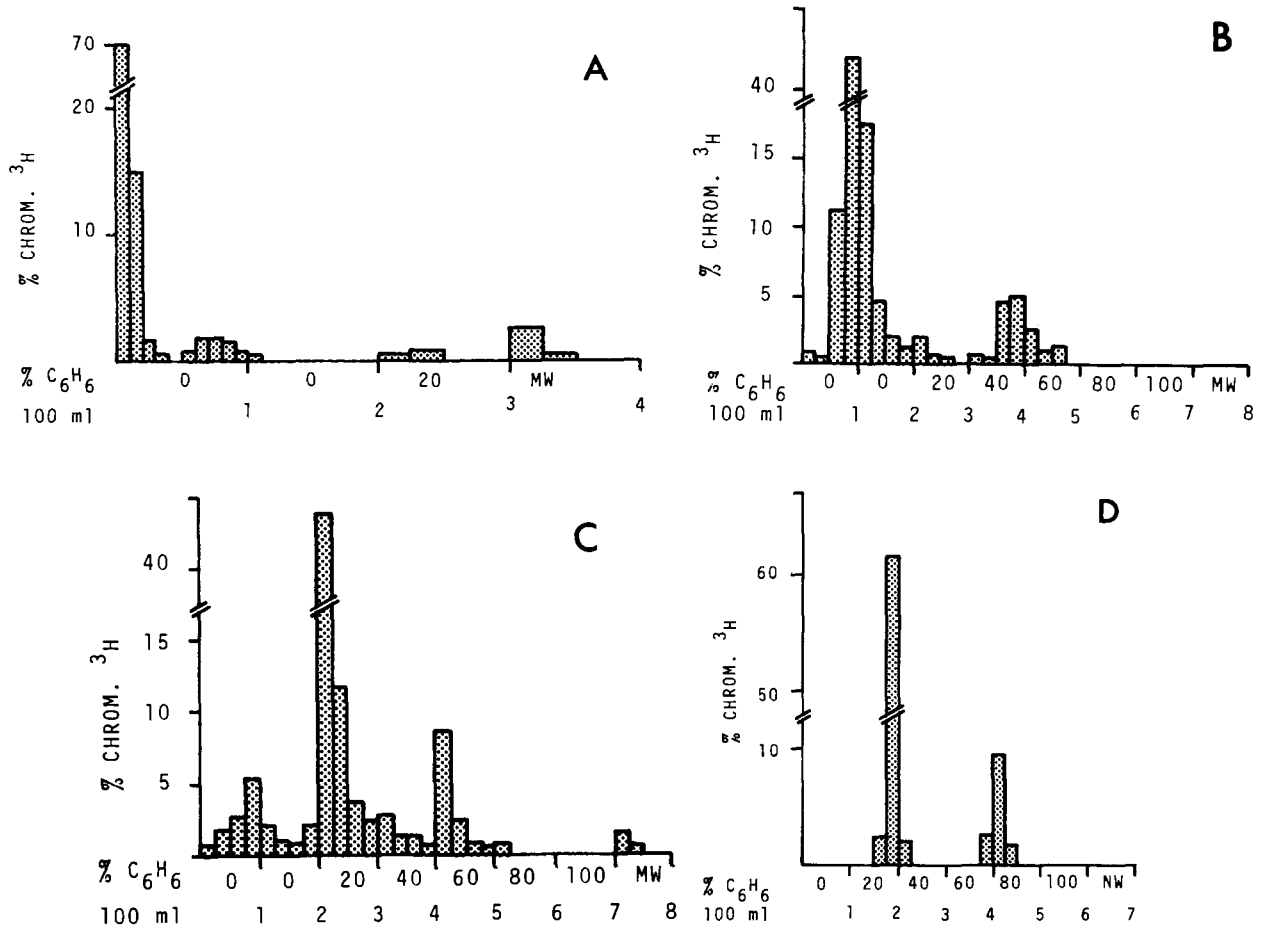

FIGURE 4: Partition chromatography of material from ethanol extracts of each of the four substrates incubated with microsomal fraction for 5 min. (A) From the radioactive diol A; (B) from the radioactive acid, B; (C) from the radioactive triol, C; (D) rechromatography of fractions 40-4 through 80-2 from incubation of allochenodeoxycholic acid.

tion chromatography (Figure 4B) elution of tritium was observed in fractions 0-3 through 0-6 (75.1%) and in fractions 40-3 through 60-1 (12.8%) corresponding to the substrate and product, respectively. Material from fractions 40-3 through 60-1 from two incubations was combined and rechromatographed and the appropriate fractions containing 92.3% of the chromatographed activity were pooled and concentrated. Half of the residue containing 2.13×10^6 dpm was mixed with 48.7 mg of authentic 3α,7α,12α-trihydroxy-5α-cholestanoic acid [mp 225–256°, lit. mp 225–226° (Kamat and Elliott, 1972), 227–229° Okuda et al., 1972; R_f 0.53 (system 1)] to give a calculated specific activity of 4.37×10^4 dpm/mg. After three crystallizations the specific activity remained constant (Table II). From the final specific activity only 49.4% of the material in these fractions was identified with 3α,7α,12α-trihydroxy-5α-cholestanoic acid, corresponding to 5.13 nmol of product formed in 5 min.

(25R)-[5α,6α-$^3H_2$]-5α-Cholestane-3α,7α,26-triol (C). The chromatogram of products obtained after incubation for 5 min (Figure 3C) showed the presence of 11.3% of chromatographed tritium in the region associated with 5α-cholestane-3α,7α,12α,26-tetrol (R_f 0.42, system 5) in addition to 54% associated with the substrate (R_f 0.76, system 5). Upon partition chromatography peaks of eluted tritium were observed (Figure 4C) in fractions 0-2 through 0-5 (12.7%), fractions 20-1 through 20-4 (61.7%), and fractions 60-1 and 60-2 (12.4%). The material in fractions 60-1 and 60-2 from two incubations was combined and rechromato-

Table II: Identification of 3α,7α,12α-Trihydroxy-5α-steroids from Incubation of 3α,7α-Dihydroxy-5α-steroids.[a]

Crystallization No. Solvent	Amount (mg)	Specific Activity ×10⁴ dpm/mg
5α-Cholestane-3α,7α,12α-triol	37.7	2.68 (Calcd)
1. Methanol	27.6 (9.2)	2.42 (3.11)
2. Methanol–acetone	16.0	2.44
3. Methanol	8.4	2.43
3α,7α,12α-Trihydroxy-5α-cholestanoic acid	48.7	4.37 (Calcd)
1. Methanol	21.6 (18.7)	3.50 (5.71)
2. Methanol–acetone	14.9	2.62 (5.28)
3. Methanol	10.8	1.92
4. Methanol	7.8	1.95
5α-Cholestane-3α,7α,12α,26-tetrol	52.5	3.59 (Calcd)
1. Methanol	37.5 (14.0)	2.91 (4.74)
2. Methanol–acetone	26.8 (9.8)	2.79
3. Methanol	21.0	2.77
3α,7α,12α-Trihydroxy-5α-cholanoic acid	43.0	1.17 (Calcd)
1. Methanol	15.2 (27.1)	1.13 (1.25)
2. Acetone–methanol	10.4	1.07
3. Methanol	8.3	1.03

[a] Identification of 5α-cholestane-3α,7α,12α-triol from 5α-cholestane-3α,7α-diol (A); 3α,7α,12α-hydroxycholestanoic acid from 3α,-7α-dihydroxy-5α-cholestanoic acid (B); 5α-cholestane-3α,7α,12α,26-tetrol from 5α-cholestane-3α,7α,26-triol (C); and allocholic acid from allochenodeoxycholic acid (D). Data for the mother liquor appear in parentheses.

Table III: Substrate Specificity of 12α-Hydroxylase.

Substrate	Relative Activity
5α-Cholestanol	0[a]
5α-Cholestane-3β,7α-ol	6[a]
3-Oxo-5α-cholestan-7α-ol	15[a]
5α-Cholestane-3α,7α-diol	100[a]
5α-Cholestane-3α,7α,26-triol	135
3α,7α-Dihydroxy-5α-cholestanoic acid	87
3α,7α-Dihydroxy-5α-cholanic acid	40

[a] Calculated from Björkhem and Gustafsson (1971).

graphed, and half of the residue (1.86×10^6 dpm) was mixed with 51.8 mg of authentic tetrol [mp 230–231°, lit. mp 230–231° (Kamat and Elliott, 1972); 232° (Hoshita, 1962); R_f 0.42 (system 5)] to give a calculated specific activity of 3.59×10^4 dpm/mg. After successive crystallizations the specific activity remained constant (2.77×10^4 dpm/mg) such that 77% of the material in these fractions was identified as 5α-cholestane-3α,7α,12α,26-tetrol, corresponding to 7.75 nmol of product.

[3β-^{3}H]Allochenodeoxycholic Acid (D). The chromatogram (Figure 3D) of products produced after 5-min incubation showed the presence of 2.28% of the tritium associated with allocholic acid. Material from two incubations was combined and chromatographed, and 18% of the tritium was spread through fractions 40-4 through 80-2. On rechromatography of these fractions (Figure 4D) 14.2% of the tritium was eluted in fractions 60-4 through 80-2, corresponding to 2.54% of the incubated tritium. Material from these fractions was combined, concentrated, and methylated, and the methyl ester (5.04×10^5 dpm in 3 mg) was mixed with 40.0 mg of methyl allocholate to give a calculated specific activity of 1.17×10^4 dpm/mg, or 1.26×10^4 dpm/mg assuming none of the 3 mg was methyl allocholate. After successive crystallizations, the specific activity was constant (1.03×10^4 dpm/mg), so 88% of material in this region was identified as allocholic acid corresponding to the formation of 1.77 nmol of product.

Discussion

These experiments demonstrate the ability of microsomal preparations of rat liver to promote 12α-hydroxylation of C_{27} and C_{24} 5α-steroids with oxygenated side chains in violation of the generalization that nuclear transformations precede side chain degradation (Bergström, 1955). Thus, 5α-cholestane-3α,7α-diol (A) was effectively converted to the corresponding 5α-cholestane-3α,7α,12α-triol as expected, but the 26-hydroxy derivative (C) of this diol (A) provided a greater quantity of 12α-hydroxylated product (Figure 2), and the corresponding 3α,7α-dihydroxy-5α-cholestan-26-oic acid was similarly oxygenated in good yield to the 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid. Lesser but measurable amounts of allocholic acid were obtained from the dihydroxy C_{24} 5α-acid, allochenodeoxycholic acid (D). Since Björkhem and Gustafsson (1971) have studied rat hepatic microsomal 12α-hydroxylation of 5α-cholestanol, 5α-cholestane-3β,7α-diol, 3-oxo-5α-cholestan-7α-ol, and 5α-cholestane-3α,7α-diol, and proposed the latter sterol as the preferred substrate for 12α-hydroxylation, a comparison of their results with those reported was made (Table III) with a relative activity of 100 assigned to 5α-cholestane-3α,7α-diol. The 26-triol (C) has a higher relative activity (135) compared to 5α-cholestane-3α,7α-diol (100) followed by the 26-oic acid (B) (87). The C_{24} dihydroxy 5α-acid, allochenodeoxycholate (D) (40) is superior to any of the other three C_{27} 5α-sterols which are apparent precursors of allochenodeoxycholate or allocholate.

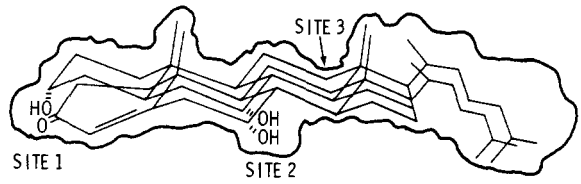

FIGURE 5: 12α-Hydroxylation of 5α-cholestane-3α,7α-diol (upper structure) or 7α-hydroxycholest-4-en-3-one (lower structure).

These results suggest that the structure of the steroid nucleus *and* the nature of the side chain are important for expression of 12α-hydroxylase activity. It is proposed that the enzyme has two active regions (site 1 and site 2) which recognize and interact with C-3 and C-7 oxygen substituents of the proper stereochemistry. The reactive center (site 3) is then properly aligned to facilitate 12α-hydroxylation of the substrate. Thus, 5α-cholestane-3α,7α-diol ideally fits these requirements, whereas the oxo group of 3-oxo-5α-cholestan-7α-ol is pointed upward and away from the site, and, accordingly, is less active at site 3. The equatorial 3β-ol of 5α-cholestan-3β,7α-diol oriented upward more than the 3-oxo group is much less active at site 3, whereas 5α-cholestan-3β-ol without the required 7α-hydroxyl group is inactive. Inspection of molecular models shows that 7α-hydroxycholest-4-en-3-one, the precursor for 12α-hydroxylation which leads to cholic acid, is virtually superimposable upon 5α-cholestane-3α,7α-diol (Figure 5). Einarsson (1968) reported the formation of about 17 nmol of product after incubation of 7α-hydroxycholest-4-en-3-one with added NADPH for 20 min, whereas Bernhardson et al. (1973) found about 5% of this product after incubation for 20 min with a reconstituted system from rat liver microsomes. The diol (A) provided 12 nmol of 5α-cholestane-3α,7α,12α-triol after incubation for 20 min in the presence of added NADPH, but not with NADH. Björkhem and Gustafsson (1971) reported a yield of 13% under similar conditions. The properties of the enzyme preparation (Table I, Figure 1) show localization in the microsomal fraction, dependency on added NADPH, and absence of requirements for metallic ions, quite similar to the properties reported by Einarsson (1968) for 12α-hydroxylation of 7α-hydroxycholest-4-en-3-one. These similarities suggest that a common enzyme is involved in these hydroxylations.

Data in Table II show that the specific activity of the mother liquor from the first crystallization of products derived from substrates A, B, and C is substantially higher than that for the crystals, indicating the presence of unidentified radioactive companions. Since the crude microsome fraction contains enzyme systems effective with NADPH for hydroxylation of steroids in many positions (Talalay, 1965; Cronholm and Johansson, 1970; Björkhem, 1972; Björkhem et al., 1973; Björkhem and Gustafsson, 1973), a number of products are possible. In the case of diol (A) 91% of the radioactive material eluted with the product was identified as 5α-cholestane-3α,7α,12α-triol, although the specific activity of the mother liquor from the first crystallization was 28% higher than the crystals. Inclusion of small amounts of 24-, 25-, or 26-hydroxylated product is possible, for Björkhem and Gustafsson (1973) showed 5β-cholestane-3α,7α-diol was hydroxylated at C-26 to about two-

thirds the extent of 5β-cholestane-3α,7α,12α-triol. The chromatographic properties of 5α-cholestane-3α,7α,25- or -3α,7α,24-triol are not known. The 26-triol (C) immediately follows 5α-cholestane-3α,7α,12α-triol in elution from the column. Because of the paucity of nonradioactive acid (B) the material in the mother liquors from the first two crystallizations of 3α,7α,12α-trihydroxy-5α-cholestanoic acid was not studied further. In the microsomal hydroxylation of 5α-cholestane-3α,7α,26-triol (C) to the tetrol the nature of contaminating products is not so clear, although it should be pointed out that 6α-hydroxylation of the 5α-cholanic acid nucleus has been suggested (Mui and Elliott, 1971b) and verified for 5α-cholestanes (Noll and Doisy, 1974). The variation in specific activity of the mother liquor from the crystals of the first crystallization of methyl allocholate is not severe, for this reaction appears to be unaccompanied by competing hydroxylations.

These studies show that a microsomal enzyme system is capable of promoting 12α-hydroxylation of steroids of appropriate nuclear configurations such that the configurations of the oxygenated substituents at C-3 and C-7 are of major importance in recognition of the substrate by the enzyme system. Similarly the constitution of the C-17 side chain must be related to this recognition by the enzyme system. Additional studies to explore these relationships are in progress.

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References

Bergström, S. (1955), *Rec. Chem. Prog. 16,* 63.
Bergström, S., and Gloor, V. (1955), *Acta Chem. Scand. 9,* 84.
Bernhardson, C., Björkhem, I., Danielsson, H., and Wikvall, K. (1973), *Biochem. Biophys. Res. Commun. 54,* 1030.
Björkhem, I. (1972), *Eur. J. Biochem. 27,* 354.
Björkhem, I., Danielsson, N., and Wikvall, K. (1973), *Eur. J. Biochem. 36,* 8.
Björkhem, I., and Einarsson, K. (1970), *Eur. J. Biochem. 13,* 174.
Björkhem, I., and Gustafsson, J. (1971), *Eur. J. Biochem. 18,* 207.
Björkhem, I., and Gustafsson, J. (1973), *Eur. J. Biochem. 36,* 201.
Cronholm, T., and Johansson, G. (1970), *Eur. J. Biochem. 16,* 373.
Danielsson, H., and Einarsson, K. (1966), *J. Biol. Chem. 241,* 1449.
Einarsson, K. (1968), *Eur. J. Biochem. 5,* 101.
Elliott, W. H. (1971), in Biochemical Applications of Mass Spectrometry, Waller, G., Ed., New York, N.Y., Wiley, p 291.
Elliott, W. H., and Hyde, P. M. (1971), *Am. J. Med. 51,* 568.
Elliott, W. H., Walsh, L. B., Mui, M. M., Thorne, M. A., and Siegfried, C. M. (1969), *J. Chromatog. 44,* 452.
Hoshita, T. (1962), *J. Biochem. 52,* 125.
Hoshita, T., Shefer, S., and Mosbach, E. H. (1968), *J. Lipid Res. 9,* 237.
Kamat, S. Y., and Elliott, W. H. (1972), *Steroids 20,* 279.
Karavolas, H. J., Elliott, W. H., Hsia, S. L., Doisy, E. A., Jr., Matschiner, J. T., Thayer, S. A., and Doisy, E. A. (1965), *J. Biol. Chem. 240,* 1568.
Matschiner, J. T., Mahowald, T. A., Elliott, W. H., Doisy, E. A., Jr., Hsia, S. L., and Doisy, E. A. (1957), *J. Biol. Chem. 225,* 771.
Mui, M. M., and Elliott, W. H. (1971a), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 30,* 1105 Abs.
Mui, M. M., and Elliott, W. H. (1971b), *J. Biol. Chem. 246,* 302.
Mui, M. M., and Elliott, W. H. (1972), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 31,* 889 Abs.
Mui, M. M., and Elliott, W. H. (1975), *Biochim. Biophys. Acta 388,* 126.
Mui, M. M., Kamat, S. Y., and Elliott, W. H. (1974), *Steroids 24,* 239.
Mui, M. M., Noll, B. W., and Elliott, W. H. (1970), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 29,* 909 Abs.
Noll, B. W., and Doisy, E. A., Jr. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 1574 Abs.
Noll, B. W., Doisy, E. A., Jr., and Elliott, W. H. (1973a), *J. Lipid Res. 14,* 385.
Noll, B. W., Doisy, E. A., Jr., and Elliott, W. H. (1973b), *J. Lipid Res. 14,* 391.
Noll, B. W., and Elliott, W. H. (1969), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 28,* 884.
Okuda, K., Horning, M. G., and Horning, E. C. (1972), *J. Biochem. 71,* 885.
Siegfried, C. M., and Elliott, W. H. (1968), *J. Lipid Res. 9,* 394.
Talalay, P. (1965), *Annu. Rev. Biochem. 24,* 347.
Wintersteiner, O., and Moore, M. (1943), *J. Am. Chem. Soc. 65,* 1503.