Bile acids. XXXIX. Metabolism of 5α-cholestane-3β,26-diol and 5α-cholestane-3β,7α,26-triol in the rat with a bile fistula

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Abstract 25R-5α-[5α,6α-$^3H_2$]Cholestane-3β,7α,26-triol was prepared from 3β,26-diacetoxy-5α[5α,6α-$^3H_2$]cholestan-7-one that was obtained from kryptogenin. Huang-Minlon reduction of the ketone provided 25R-5α-[5α-^{3}H]cholestane-3β,26-diol. Results from mass spectrometry, molecular rotation, and several types of chromatography are consonant with the assigned structures. Bile was collected for 8 days from adult male rats, with cannulated bile ducts, that had received approximately 0.8 mg of the triol or diol intraperitoneally. Bile from the first 12 hr was hydrolyzed, and the bile acids were separated by partition chromatography. The chromatographic pattern of separated bile acids was much simpler for the triol than the diol. Approximately 50% of the bile acids derived from the triol were trihydroxy allo acids (allocholic acid, 44%, and its 3β isomer, 5.3%); only 16.4% allocholic acid was obtained from the diol. Comparable amounts of allochenodeoxycholic acid were derived from the diol and triol (21.2% and 28.2%, respectively). Unidentified metabolites in the dihydroxy acid fraction derived from the diol constitute 15.8% of chromatographed material.

Supplementary key words 25R-5α-[5α,6α-$^3H_2$]cholestane-3β,7α,26-triol · 25R-5α-[5α-^{3}H]cholestane-3β,26-diol · allocholic acid · allochenodeoxycholic acid · 3β-hydroxy allo bile acids · kryptogenin · mass spectrometry · gas–liquid chromatography

Earlier reports from this laboratory have shown that allocholic and allochenodeoxycholic acids and their 3β isomers are the principal acidic metabolites of 5α-cholestanol in the normal and in the hyperthyroid rat (1–3). 5α-Cholestane-3β,7α-diol has also been shown to be converted to these metabolites by the rat with a cannulated bile duct, but in different quantities compared with those derived from 5α-cholestanol (4). In order to ascertain the effect of addition of a C-26 hydroxyl group to 5α-cholestanol and to 5α-cholestane-3β,7α-diol on the quantities of these metabolites produced by the rat with a bile fistula, 5α-[5α-^{3}H]cholestane-3β,26-diol and 5α-[5α,6α-$^3H_2$]5α-cholestane-3β,7α,26-triol were prepared from kryptogenin, and the results of in vivo studies are reported in this paper. A preliminary report of some of these studies has appeared (5).

METHODS AND MATERIALS

Bile acids were separated by acetic acid partition chromatography (6). The fractions have been designated according to the percentage of benzene in hexane; e.g., fraction 40-3 represents the third fraction of the eluent containing 40% benzene in hexane. Analytical TLC, PLC, and GLC were carried out as described previously (2, 7). The hydroxysteroids were converted to their TMSi ethers for GLC (7), and their relative retention times (RRT) were related to cholestane on 3% OV-17 (RRT = 1.00; absolute time = 5.28 min). Mass spectra were determined with the direct inlet probe; ion source, 230°C; ionizing energy, 70 eV; accelerating voltage, 3.5 kV (8). Radioactivity was determined in Bray's solution as described previously (3). Melting points were

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; PLC, preparative-layer chromatography; TMSi, trimethylsilyl; RRT, relative retention time. Systematic nomenclature of the compounds referred to in the text by their trivial names is as follows: cholestanol, 5α-cholestan-3β-ol; allolithocholic acid, 3α-hydroxy-5α-cholan-24-oic acid; allochenodeoxycholic acid, 3α,7α-dihydroxy-5α-cholan-24-oic acid; allocholic acid, 3α,7α,12α-trihydroxy-5α-cholan-24-oic acid; all 5α-cholanic acids are 5α-cholan-24-oic acids.

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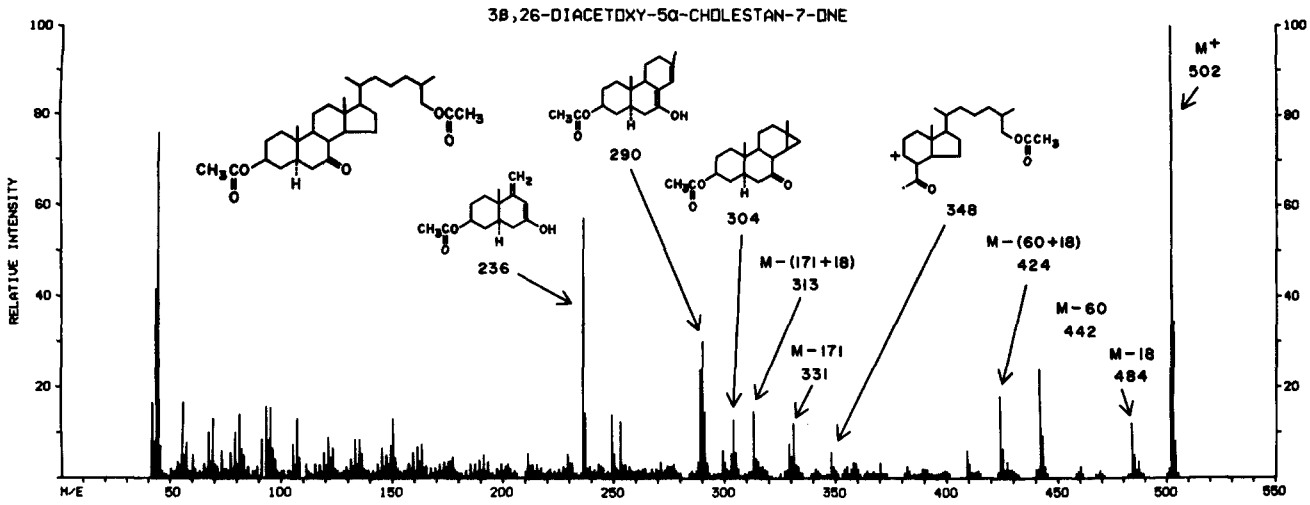

FIG. 1. Mass spectrum of 3β,26-diacetoxy-5α-cholestan-7-one. The temperature of the direct probe was 70°C.

determined on a Fisher-Johns apparatus and are reported as read. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. Rotations were determined (4) in 1% chloroform; infrared spectra were determined in Nujol (2). Allo bile acids were obtained from appropriate methyl cholanates (9).

Preparation of tritiated sterols

3β,26-Diacetoxycholest-5-en-7-one. Cholest-5-ene-3β,26-diol diacetate was prepared from kryptogenin (3β,26-dihydroxycholest-5-ene-16,22-dione) (Syntex, S.A., Mexico City) by a modification of the method of Scheer, Thompson, and Mosettig (10); mp 128–129°C, $[\alpha]_D^{28} = -38°$; reported mp 128–129°C, $[\alpha]_D^{28} = -35°$ (10). The diacetate (1 g) was oxidized with chromium trioxide according to Fieser (11) to provide 414 mg of impure ketone. PLC of the product in benzene–ethyl acetate 9:1 followed by crystallization of the material from the main band from hexane afforded 350 mg (34% yield) of long needles, mp 120–122°C. Multiple crystallizations from methanol and from hexane gave 3β,26-diacetoxycholest-5-en-7-one, pure by TLC; mp 121–122°C; UV max (C_2H_5OH) 234 nm (log ϵ = 4.12); ν_{max} 1736 (—$OCOCH_3$), 1678 (—C=C—C=O), 1637, 1335, 1316, 1299, 1287, 1245 (—$OCOCH_3$), 1241 (—$OCOCH_3$), 1208, 1185, 1133, 1117, 1033, 978, 950, 935, 918, 902, and 867 cm^{-1}; R_F 0.29 (benzene–ethyl acetate 9:1); the mass spectrum showed a small molecular ion, *m/e* 500, base peak at *m/e* 440 (M − 60), and other recognizable fragments at *m/e* 425 [M − (60 + 15)], 380 [M − (2 × 60)], 269 [M − (60 + side chain)], 242, and 174 (8).

Analysis: $C_{31}H_{48}O_5$; calculated: C, 74.36; H, 9.66
found: C, 74.61; H, 9.69

3β,26-Diacetoxy-5α-cholestan-7-one. Reduction of the above ketone (350 mg) in 30 ml of ethyl acetate with hydrogen and 56 mg of platinum oxide at 2 atm for 30 min provided a mixture that contained three major components as seen by TLC in benzene–ethyl acetate 9:1. Purification by PLC gave 121 mg (R_F 0.11), corresponding to the 7β-ol (34%); 225 mg (R_F 0.28) corresponding to the 5α-7-ketone (64%); and 14 mg (R_F 0.74) corresponding to 5α-cholestane-3β,26-diol diacetate (4%). Crystallization of the material from the center band from methanol gave 184 mg of 3β,26-diacetoxy-5α-cholestan-7-one: mp 126–127°C; ν_{max} 1739 (—$OCOCH_3$), 1706 (C=O), 1242 (—$OCOCH_3$), 1176, 1152, 1131, 1109, 1078, 1044, 1027, 996, 975, 952, 942, 912, 900, and 890 cm^{-1}; the mass spectrum is given in Fig. 1.

Analysis: $C_{31}H_{50}O_5$; calculated: C, 74.06; H, 10.03
found: C, 74.30; H, 10.20

Alkaline hydrolysis of the diacetate provided 3β,26-dihydroxy-5α-cholestan-7-one. An analytical sample was obtained as needles from methanol; mp 176–178°C; R_F 0.37 in benzene–ethyl acetate 2:3; ν_{max} 3268 (—OH), 1704 (C=O), 1300, 1287, 1259, 1227, 1221, 1193, 1174, 1126, 1080, 1074 (—OH), 1063, 1031 (—OH), 1020, 985, 962, 951, 937, 923, 905, 864, 853, and 819 cm^{-1}; RRT of the TMSi ether, 10.15. The mass spectrum showed molecular ion *m/e* 418, as base peak, *m/e* 400 (M − 18), and other identifiable fragments at 306, 289, 271, 262, 248, and 194, characteristic of a saturated 7-oxo sterol (8).

Analysis: $C_{27}H_{46}O_3$; calculated: C, 77.46; H, 11.08
found: C, 77.61; H, 11.17

The 5α-cholestane-3β,7β,26-triol 3,26-diacetate (R_F 0.42)

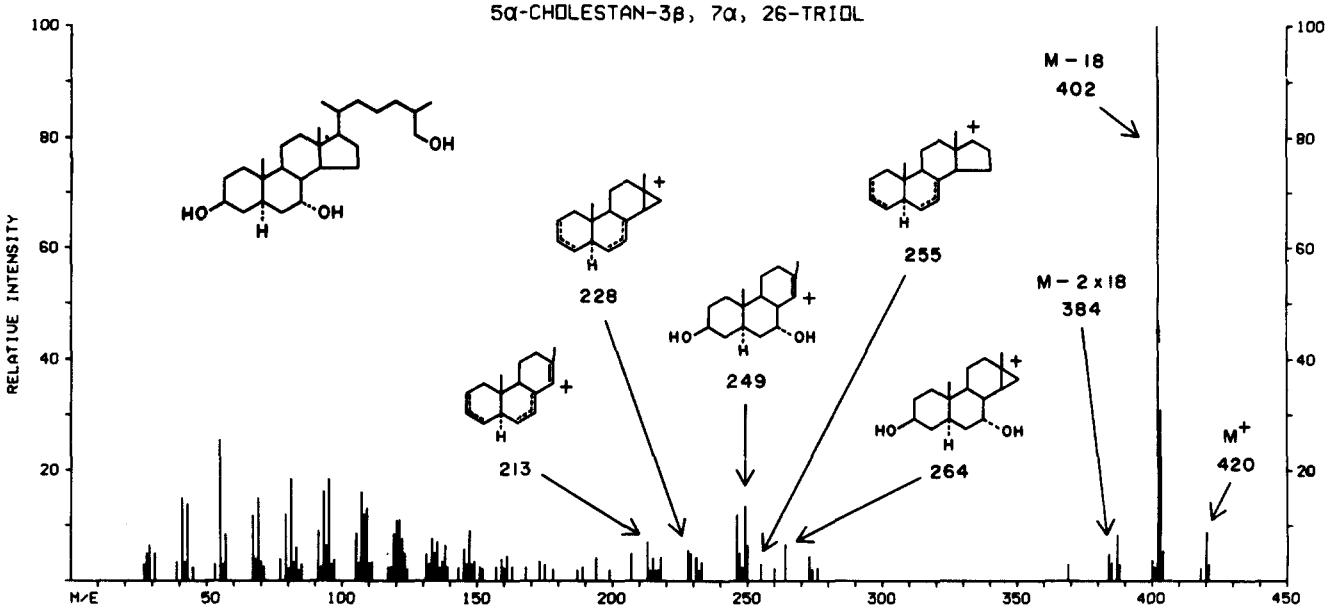

FIG. 2. Mass spectrum of 5α-cholestane-3β, 7α,26-triol. The temperature of the direct probe was 66°C.

was purified by PLC in 2,2,4-trimethylpentane–propan-2-ol 85:15, but the pure material was an oil that resisted crystallization. Hydrolysis of the oil with 4% methanolic potassium hydroxide at 60°C for 1 hr provided 5α-cholestane-3β,7β,26-triol; crystallization from acetone afforded fine needles: mp 201°C; $[\alpha]_D^{23}$ = +62°; ν_{max} 3356 (OH), 3215 (OH), 3145 (OH), 1340, 1309, 1277, 1248, 1217, 1200, 1171, 1157, 1115, 1079, 1041, 1024, 993, 936, 930, 900, 862, and 733 cm^{-1}; RRT of TMSi ether, 5.63.

Analysis: $C_{27}H_{48}O_3$; calculated: C, 77.09; H, 11.50
found: C, 76.92; H, 11.45

5α-Cholestane-3β,7α,26-triol. Reduction of 251 mg of 3β,26-diacetoxy-5α-cholestan-7-one with excess sodium borohydride in methanol provided a product that showed two main spots (R_F 0.42 and 0.55) on TLC in 2,2,4-trimethylpentane–propan-2-ol 85:15. The smaller, less polar spot corresponded to 5α-cholestane-3β,7β,-26-triol 3,26-diacetate. The more polar material, 5α-cholestane-3β,7α,26-triol 3,26-diacetate, was obtained as an oil (154 mg) pure by TLC, after PLC with the above solvent mixture. The mass spectrum showed a small molecular ion, *m/e* 504, base peak at *m/e* 486 (M − 18), and fragments at 426 [M − (60 + 18)], 306, 291, 255, 228, and 213.

Alkaline hydrolysis of the oil (138 mg) afforded a product that was recrystallized from acetone to give fine needles of 5α-cholestane-3β,7α,26-triol; mp 206–207°C; $[\alpha]_D^{23}$ = +12.6°; RRT of the TMSi ether, 3.82; R_F 0.18 in acetone–benzene 30:70; ν_{max} 3268 (OH), 1337, 1295, 1239, 1215, 1160, 1133, 1116, 1100, 1087, 1045, 1031, 1016, 989, 953, 941, 913, 887, 845, and 810 cm^{-1}; the mass spectrum is shown in Fig. 2.

Analysis: $C_{27}H_{48}O_3$; calculated: C, 77.09; H, 11.50
found: C, 76.81; H, 11.29

3β,26-Diacetoxy-5α-[5α,6α-$^3H_2$]cholestan-7-one. Catalytic reduction of 3β,26-diacetoxy cholest-5-en-7-one with 3 Ci of tritium gas was performed by New England Nuclear Corp. After addition of 10.0 mg of 5α-cholestane-3β,26-diol diacetate (containing some cholest-5-ene-3β,26-diol diacetate), 100.5 mg of 3β,26-diacetoxy-5α-cholestan-7-one, and 68.8 mg of a mixture of the 3,26-diacetates of 5α-cholestane-3β,7β,26-triol and the 3β,7α,26-triol to an aliquot of the tritiated material (93.2 mCi), the desired 3β,26-diacetoxy-5α-[5α,6α-$^3H_2$]-cholestan-7-one (102 mg) was separated by PLC in benzene–ethyl acetate 9:1. Purification by repeated PLC provided 76.2 mg with a specific activity of 1.56 × 10^9 dpm/mg. Crystallization from methanol raised the specific activity slightly to 1.60 × 10^9 dpm/mg. Three crystallizations did not lower the specific activity.

Location of ^3H in 3β,26-diacetoxy-5α-[5α,6α-$^3H_2$]-cholestan-7-one. The tritiated compound (0.056 mg, sp act 1.60 × 10^9 dpm/mg.) was diluted with 20.52 mg of the unlabeled compound and was crystallized from methanol to provide 16.0 mg with specific activity of 4.37 × 10^6 dpm/mg (2.19 × 10^9 dpm/mmole; calcd sp act 2.18 × 10^9 dpm/mmole). The diluted material, 15.2 mg, was refluxed for 1 hr in 2.5 ml of 1.25 N sodium hydroxide in 70% methanol (12). From the hydrolysate, 12.3 mg of 3β,26-dihydroxy-5α-[5α-^3H]cholestan-7-one was obtained, pure by TLC in benzene–ethyl

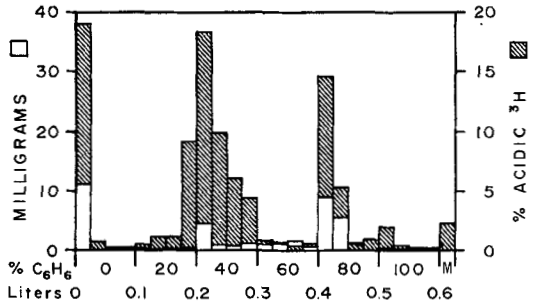

FIG. 3. Partition chromatography of the acidic fraction derived from bile obtained from rat E in the first 12 hr after the administration of 5α-[5α-³H]cholestane-3β,26-diol. The heights of the open bars *from the base line* indicate the amounts of mass in the fractions; the heights of the hatched bars *from the base line* denote the amounts of tritium in the fractions. *M*, methanol.

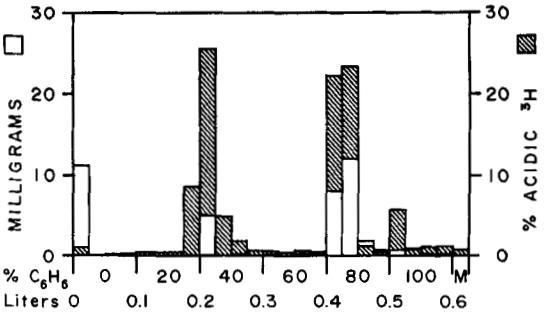

FIG. 4. Partition chromatography of the acidic fraction derived from bile obtained from rat H in the first 12 hr after the administration of 5α-[5α,6α-³H₂]cholestane-3β,7α,26-triol. Explanations of bar heights and *M* are given in legend to Fig. 3.

acetate 2:3. After crystallization from benzene, 10.4 mg of crystals had a specific activity of 3.62×10^6 dpm/mg (1.52×10^9 dpm/mmole). On a molar basis, 69% of the tritium originally in the molecule was retained. Beugelmans et al. (12) employed similar conditions to enolize 5α-[6,6,8β-²H₃]androstan-7-one (²H₂, 6%; ²H₃, 94%) to the [8β-²H₁]compound (²H₀, 6%; ²H₁, 93%). Thus, the ease of replacement of the enolizable hydrogens of the 7-ketone is 6α and 6β ≫ 8β, and the 31% of tritium lost from 3β,26-diacetoxy-5α-[5α,6α-³H₂]cholestan-7-one was derived predominantly or completely from C-6.

5α-[5α,6α-³H₂]Cholestane-3β,7α,26-triol. Reduction of the tritiated 3β,26-diacetoxy-5α-cholestan-7-one (50 mg, sp act 1.60×10^9 dpm/mg) with sodium borohydride in the usual manner provided a product that was subjected to repeated PLC in 2,2,4-trimethylpentane–propan-2-ol 82.5:17.5 to give 24.1 mg of 5α-[5α,6α-³H₂]cholestane-3β,7α,26-triol 3,26-diacetate; analysis of 100 μg by TLC in 2,2,4-trimethylpentane–propan-2-ol 85:15 showed a single spot (R_F 0.50; R_F of the 7β-ol, 0.44). TLC of 8 μg on Supelcosil 12B in 2,2,4-trimethylpentane–propan-2-ol 92:8 gave one spot (R_F 0.64, detected with iodine vapor), which contained at least 98.4% of the tritium. Only 0.1% of the radioactivity corresponded to the 7β-ol (R_F 0.53). The diacetate was hydrolyzed under reflux with 4% methanolic potassium hydroxide for 1 hr and allowed to stand overnight. Crystals of 5α-[5α,6α-³H₂]cholestane-3β,7α,26-triol had a specific activity of 1.74×10^9 dpm/mg. Three crystallizations from acetone and two from methanol gave 7.8 mg with no significant change in specific activity. The average value was 1.75×10^9 dpm/mg.

5α-[5α-³H]Cholestane-3β,26-diol. Huang-Minlon reduction (13) of 3β,26-diacetoxy-5α-[5α, 6α-³H₂]cholestan-7-one (sp act 1.60×10^9 dpm/mg; 8.03×10^{11} dpm/mmole) provided material that showed a predominant spot (R_F 0.44) on TLC in benzene–ethyl acetate 45:55 corresponding to authentic cholestane-3β,26-diol (10). Purification by repeated PLC in the above solvent mixture provided 13.5 mg of material that showed one spot on analytical TLC on Supelcosil 12B in benzene–ethyl acetate 3:1 (R_F 0.38) and contained at least 98% of the radioactivity. The residue was crystallized from methanol, benzene, and twice from methanol to provide 5.9 mg of 5α-[5α-³H]cholestane-3β,26-diol with a specific activity of 1.32×10^9 dpm/mg (5.32×10^{11} dpm/mmole). This specific activity showed that 66.2% of the tritium of the reactant was retained, in agreement with 69% retention after enolization of the ketone for 1 hr in 1.25 N sodium hydroxide. It should be noted that Shapiro et al. (14) reduced 3β-acetoxyandrost-4-en-7-one with deuterium, and converted the product to 5α-[5α-²H]androstan-3β-ol via the Huang-Minlon reaction as was used in our preparation of 5α-[5α-³H]cholestane-3β,26-diol.

To relate the tritiated material with nonradioactive 5α-cholestane-3β,26-diol, an aliquot of a solution containing 0.127 mg of the above crystalline material was diluted with 52.53 mg of synthetic 5α-cholestane-3β,26-diol (mp 180–181°C; $[\alpha]_D^{19} = +28°$; R_F 0.69 on Adsorbosil-1 in benzene–ethyl acetate 45:55; reported (10) mp 179–180°C, $[\alpha]_D^{20} = +28°$). After three successive crystallizations from methanol and three from benzene, the remaining crystalline material (28.6 mg) was acetylated and the acetate was crystallized three times from methanol and twice from hexane. Through these 11 crystallizations the specific activity varied less than 4%, confirming the identity of the tritiated material with the nonradioactive sterol.

Animal experiments

Six male rats, age 13 wk, were prepared with bile fistulas and allowed to recover 55–59 hr before intraperitoneal administration of the sterols. The sterol

(about 1 mg) was dissolved in 0.2 ml of 95% ethanol, 4 drops of Tween 80 were added, and the solutions were diluted with water to 1 ml; 0.83 mg of 5α-[5α-^{3}H]cholestane-3β,26-diol was administered to rat E (285 g), 0.84 mg to rat F (240 g), and 0.83 mg to rat G (385 g); 0.85 mg of 5α-[5α,6α-$^3H_2$]cholestane-3β,7α,26-triol was given to rat H (265 g), 0.81 mg to rat I (275 g), and 0.86 mg to rat J (325 g). Bile was collected every 12 hr for the first 3 days and every 24 hr thereafter for 8 days and assayed for tritium. Recovery of injected tritium after 12 hr was: rat E, 26.2%; rat F, 24.6%; rat G, 70.5%; rat H, 62%; rat I, 23.2%; rat J, 23.4%. Bile from the first 12 hr was hydrolyzed and fractionated (3), and the following distribution of biliary tritium (rats E and H, respectively) was found: acidic fraction, 75.5%, 72.6%; water wash of acidic fraction, 6.4%, 10.5%; nonsaponifiable fraction, 0.3%, 0.2%; water wash of nonsaponifiable fraction, 0.4%, 0.6%; and hydrolyzed, extracted bile, 8.6%, 7.5%. Comparable results were obtained from rats F, G, and I; 90% of the ^{3}H appeared in the acid fraction from rat J. The acid fractions were chromatographed on acetic acid partition columns; the chromatogram from rat E is shown in Fig. 3 and that from rat H is shown in Fig. 4. The mass associated with fractions 0-1, 40-1, 60-3, 4 and 80-1, 2 has been shown (6) to contain fatty acids, chenodeoxycholic acid, β-muricholic acid, and cholic acid, respectively.

RESULTS

Metabolism of 5α-[5α-^{3}H]cholestane-3β,26-diol

Investigation of metabolites in fractions 0-1 and 0-2. An aliquot ($^2/_5$) of the material in fractions 0-1 and 0-2 (Fig. 3) was treated with diazomethane. Methyl allolithocholate (21.11 mg) and methyl 3β-hydroxy-5α-cholanate (41.83 mg) were added, and the mixture was fractionated by PLC in benzene–acetone 9:1. A strip 2 cm wide was removed from one plate in nine individual bands with assay of tritium as follows: band 1 (top 4.5 cm), 1% ^{3}H; band 2 (next 2.4 cm), 10%; band 3 (1.3 cm), 5%; band 4 (1.2 cm, corresponding to methyl allolithocholate, R_F 0.49), 6%; band 5 (0.7 cm), 2%; band 6 (1.1 cm, corresponding to methyl 3β-hydroxy-5α-cholanate, R_F 0.38), 41%; band 7 (2.1 cm), 6%; band 8 (3.2 cm), 10%; and band 9 (1.5 cm including the origin), 18%. The two appropriate bands were scraped from the plates and eluted to provide 20.5 mg corresponding to methyl allolithocholate and 39.3 mg corresponding to the 3β isomer. Methyl allolithocholate exhibited a specific activity of 2.87 × 10^4 dpm/mg after three crystallizations from acetone (Table 1). Thus, allolithocholic acid accounted for 1.4% of the tritium in the bile acid fraction. After three successive crystallizations, methyl 3β-hydroxy-5α-cholanate had a specific activity of 1.22 × 10^5 dpm/mg (Table 1), but the successive values were still decreasing. Consequently, no more than 5.9% of the biliary tritium could be present in 3β-hydroxy-5α-cholanic acid. Unidentified metabolites in fractions 0-1 and 0-2 constitute about 12% of the biliary acidic metabolites.

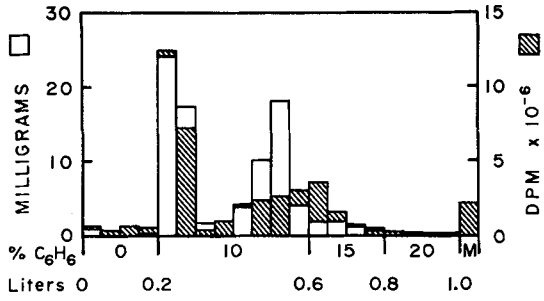

FIG. 5. Partition chromatography of two-fifths of the combined material from fractions 20-2 through 60-1 (Fig. 3) after methylation and isotopic dilution with methyl 3α,7α- and 3β,7α-dihydroxy-5α-cholanates. Explanations of bar heights and *M* are given in legend to Fig. 3.

Investigation of metabolites in fractions 20-2 through 60-1. The residue from fractions 20-2 through 60-1 (9 mg, 1.10 × 10^8 dpm of ^{3}H) (Fig. 3) was combined, and an aliquot ($^2/_5$) was removed for treatment with diazomethane. After evaporation of the solvent, 42.50 mg of methyl allochenodeoxycholate and 45.36 mg of methyl 3β,7α-dihydroxy-5α-cholanate were added. The mixture was chromatographed (Fig. 5), and the residue from fractions 10-1 and 10-2, which corresponded to methyl allochenodeoxycholate (41.5 mg), was combined and crystallized four times to constant specific activity,

TABLE 1. Isotopic dilution of the metabolites from fractions 0-1 and 0-2 (Fig. 3) with methyl 3α- and 3β-hydroxy-5α-cholanates[a]

Crystallization	Weight of Crystals	Specific Activity
	mg	*dpm/mg* × 10^{-4}
Methyl allolithocholate		
1. Acetone	14.5	2.70
2. Acetone	9.7	2.98
3. Acetone	3.4	2.87
Methyl 3β-hydroxy-5α-cholanate		
1. Acetone	28.2	14.0
2. Acetone	19.5	13.2
3. Acetone	16.6	12.2

[a] An aliquot ($^2/_5$) of the combined fractions 0-1 and 0-2, containing 4.5 mg and 1.61 × 10^7 dpm, was methylated with diazomethane, and 21.11 mg of methyl 3α-hydroxy-5α-cholanate and 41.83 mg of methyl 3β-hydroxy-5α-cholanate were added. The combined materials were separated by TLC before crystallization.

TABLE 2. Identification of methyl allochenodeoxycholate and methyl 3β,7α-dihydroxy-5α-cholanate by isotopic dilution of the methylated metabolites from fractions 20-4 to 60-1 (Fig. 5)

Crystallization No. and Solvent	Weight of Crystals	Specific Activity
	mg	*dpm/mg* × 10^{-5}
Methyl 3α,7α-dihydroxy-5α-cholanate		
Calculated[a]	41.5	4.72
1. Acetone–water	20.6	4.45
2. Acetone–water	18.4	4.33
3. Acetone–water	16.7	4.32
4. Methanol–water	13.1	4.32
Methyl 3β,7α-dihydroxy-5α-cholanate		
Calculated[b]	15.6	1.19
1. Acetone	14.5	0.94
2. Acetone	10.6	0.86
3. Acetone	8.9	0.93
4. Methanol–water	5.2	0.86
After dilution and chromatography		
Calculated[c]	13.76	0.189
1. Acetone	13.0	0.182
2. Acetone	5.7	0.184
3. Acetone	3.2	0.184

[a] Fractions 10-1 and 10-2 (Fig. 5, 41.5 mg, 1.96 × 10^7 dpm) were combined for crystallization.

[b] Fractions 10-4 and 10-5, which contained 15.6 mg and 1.84 × 10^5 dpm, were combined for crystallization.

[c] An additional 10.74 mg of methyl 3β,7α-dihydroxy-5α-cholanate was added to 3.022 mg of material from the fourth crystallization before partition chromatography. After collection of eight hexane fractions, fractions 10-1 to 10-5 from this column were combined for crystallization.

4. 32 × 10^5 dpm/mg (Table 2). Thus, allochenodeoxycholic acid represents at least 21.2% of the radioactivity in the biliary acid fraction.

Because the tritium in the region of methyl 3β,7α-dihydroxy-5α-cholanate had not eluted coincidentally with mass, fractions 10-5 through 10-7 (32 mg, 7.0 × 10^6 dpm, Fig. 5) were rechromatographed to remove the metabolite from the tritium in subsequent fractions 10-8 to 15-3. The residue from fractions 10-5 through 10-7 from this column was rechromatographed; material from the appropriate fractions (10-4, 10.1 mg, 1.26 × 10^6 dpm, and 10-5, 5.4 mg, 0.58 × 10^6 dpm) was combined, and the residues were crystallized four times to provide a specific activity of 0.86 × 10^5 dpm/mg (Table 2). Because some variation persisted in this specific activity, a sample of the fourth crop of crystals (3.022 mg) was diluted with 10.74 mg of authentic methyl 3β,7α-dihydroxy-5α-cholanate, and the mixture was rechromatographed with coincident elution of mass and radioactivity. Mass from fractions 10-1 to 10-5 was combined and crystallized (Table 2) successively from acetone without significant change in specific activity. On the basis of the final specific activity, 3β,7α-dihydroxy-5α-cholanic acid represents at least 4.4% of the tritium in the biliary acid fraction.

By rechromatography of the unidentified tritiated metabolites in fractions 10-3 to 15-4 (Fig. 5) it can be shown that at least two additional metabolites are present. The less polar material (fractions 10-3 to 10-7) represents 5.9% and the more polar material (fractions 10-8 to 15-4) represents 9.9% of the radioactivity in the biliary acid fraction. The nature of these metabolites is now under investigation.

Investigation of metabolite in fractions 80-1 and 80-2. An aliquot (2/5, 5.8 mg, 1.70 × 10^7 dpm) of the material in fractions 80-1 and 80-2 (Fig. 3) was treated with diazomethane, the residue was diluted with 48.42 mg of synthetic methyl allocholate, and the mixture was chromatographed. Residue in fractions 40-3, 40-4, and 60-1, which showed coincident elution of mass and radioactivity, was crystallized to constant specific activity (Table 3). Material from the fourth crop of crystals and the mother liquors was subjected to repeated PLC on Supelcosil 12B (cyclohexane–ethyl acetate–acetic acid

TABLE 3. Isotopic dilution of the methylated metabolite from fractions 80–1 and 80–2 (Fig. 3)

Crystallization No. and Solvent	Weight of Crystals	Specific Activity
	mg	*dpm/mg* × 10^{-5}
Calculated value[a]	48.42	3.51
1. Acetone–hexane	40.7	2.93
2. Acetone–hexane	23.9	3.01
3. Acetone	14.0	2.92
4. Acetone	8.6	2.98
After PLC of mother liquors and fourth crystals to remove methyl cholate (TLC-pure)		
1. Acetone	7.9	2.93

[a] TLC-pure methyl 3α,7α,12α-trihydroxy-5α-cholanate, 48.42 mg, was added to a methylated aliquot (2/5) of the combined fractions 80-1 and 80-2 (Fig. 3), containing 1.70 × 10^7 dpm of ^{3}H and 5.8 mg, and the mixture was chromatographed before crystallization. The calculation assumes the weight of methyl 3α,7α,12α-trihydroxy-5α-cholanate in the aliquot to be negligible.

TABLE 4. Isotopic dilution of the methylated metabolite from fractions 80-4, 100-1, and 100-2 (Fig. 3) with methyl 3β,7α,12α-trihydroxy-5α-cholanate

Crystallization No. and Solvent	Weight of Crystals	Specific Activity
	mg	*dpm/mg* × 10^{-4}
Calculated value[a]	48.02	4.85
After partition chromatography		
1. Acetone	37.8	3.57
2. Acetone	28.0	3.38
3. Acetone	20.7	3.31

[a] Methyl 3β,7α,12α-trihydroxy-5α-cholanate (48.02 mg) was added to a methylated aliquot (2/5) of the combined fractions 80-4, 100-1, and 100-2, containing 2.33 × 10^6 dpm/mg and 0.4 mg, and the mixture was chromatographed before crystallization.

7:23:3) (15) to provide a product free of methyl cholate as shown by analytical TLC. The product crystallized from acetone to provide a specific activity of 2.93 × 10^5 dpm/mg (Table 3), indicating that allocholic acid accounts for 16.4% of the tritium in the biliary acid fraction.

Investigation of metabolite in fractions 80-4 to 100-2. An aliquot ($^2/_5$) of the material in fractions 80-4, 100-1, and 100-2 (Fig. 3) was treated with diazomethane and diluted with authentic methyl 3β,7α,12α-trihydroxy-5α-cholanate (48.02 mg). The mixture was chromatographed, and material in fractions 60-1 to 60-4 was combined and crystallized from acetone (Table 4). The specific activity showed little variation after the second and third crystallizations; from these data, 3β,7α,12α-trihydroxy-5α-cholanic acid represents at least 1.8% of the radioactivity in the biliary acid fraction.

Metabolism of 5α-[5α,6α-$^3H_2$]cholestane-3β,7α,26-triol

Investigation of metabolites in fractions 20-4 through 40-3. Fractions 20-4 through 40-3 (Fig. 4), containing 5.0 mg and 2.72 × 10^8 dpm, were combined, and an aliquot ($^2/_5$) was removed for treatment with diazomethane. Methyl allochenodeoxycholate (48.44 mg) and 48.24 mg of methyl 3β,7α-dihydroxy-5α-cholanate were added to the radioactive material and chromatographed.

Radioactivity was eluted coincidentally with the two mass peaks (Fig. 6). The contents of fractions 10-1 and 10-2 (methyl allochenodeoxycholate, 50 mg, 7.82 × 10^7 dpm) were combined and crystallized twice from acetone–water to give a final specific activity of 1.55 × 10^6 dpm/mg. Thus, methyl allochenodeoxycholate represents 76% of the diluted sample, and allochenodeoxycholic acid represents at least 28.2% of the radioactivity in the biliary acid fraction. The contents of fractions 10-5 to 10-8 (methyl 3β,7α-dihydroxy-5α-cholanate, 45.4 mg, 1.12 × 10^7 dpm, 2.47 × 10^5 dpm/mg), were combined and crystallized twice from acetone to give a final specific activity of 2.36 × 10^5 dpm/mg. Thus, methyl 3β,7α-dihydroxy-5α-cholanate represents 12% of the diluted sample, and 3β,7α-dihydroxy-5α-cholanic acid represents at least 4.3% of the tritium in the biliary acid fraction. The ratio of 3α,7α- to 3β,7α-dihydroxy-5α-cholanic acid formed is 6.6.

Investigation of metabolites in fractions 80-1 through 80-3. Fractions 80-1 through 80-3 (Fig. 4), containing 21.8 mg and 3.10 × 10^8 dpm, were combined. An aliquot ($^2/_5$, 8.7 mg, 1.24 × 10^8 dpm) was removed and methylated with diazomethane. After 44.63 mg of methyl allocholate was added, the diluted sample was chromatographed. Fractions 40-1 to 60-1 were combined, and a specific activity of 2.57 × 10^6 dpm/mg was obtained with no significant change after three crystallizations. The three mother liquors and the third crop of crystals were recombined and subjected to repeated PLC on Supelcosil

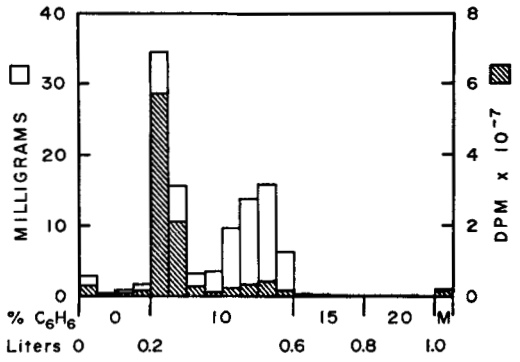

FIG. 6. Partition chromatography of two-fifths of the combined material from fractions 20-4 through 40-3 (Fig. 4) after methylation and dilution with methyl 3α,7α- and 3β,7α-dihydroxy-5α-cholanates. Explanations of bar heights and *M* are given in legend to Fig. 4.

12B in cyclohexane–ethyl acetate–acetic acid 7:23:3 to remove methyl cholate. A sample (6.7 mg) that showed only one spot on TLC was crystallized from acetone to provide a slightly higher specific activity, 2.64 × 10^6 dpm/mg. Therefore, at least 89% of the radioactivity in the diluted sample is methyl allocholate, and 44% of the tritium in the biliary acid fraction is associated with allocholic acid.

Investigation of metabolites in fractions 100-1 and 100-2. Fractions 100-1 and 100-2 (Fig. 4), which contained 1.6 mg and 4.30 × 10^7 dpm, were combined, and $^2/_5$ of the total residue was methylated and diluted with 46.03 mg of methyl 3β,7α,12α-trihydroxy-5α-cholanate. Mass and radioactivity were eluted coincidentally in partition chromatography. Fractions 40-5 to 60-3 were combined and crystallized three times from acetone; the specific activity (3.07 × 10^5 dpm/mg) varied less than 2% for the three crystallizations. Methyl 3β,7α,-12α-trihydroxy-5α-cholanate contained 82% of the isotopically diluted sample, and 3β,7α,12α-trihydroxy-5α-cholanic acid accounts for 5.3% of the acidic biliary radioactivity. The ratio of 3α,7α,12α- to 3β,7α,12α-trihydroxy-5α-cholanic acid formed from 5α-[5α,6α-$^3H_2$]cholestane-3β,7α,26-triol is 8.3.

DISCUSSION

5α-Cholestane-3β,26-diol and 3β,7α,26-triol were prepared from kryptogenin via the intermediate cholest-5-ene-3β,26-diol diacetate. Allylic oxidation provided 3β,26-diacetoxycholest-5-en-7-one, which exhibited an absorption maximum at 234 nm (log ε = 4.12) comparable to that of 3β-acetoxycholest-5-en-7-one (log ε = 4.12 at 235 nm) (4). The mass spectrum confirmed the molecular weight and showed a fragmentation pattern comparable to 3β-acetoxycholest-5-en-7-one except for the 26-acetoxyl group; thus, both spectra exhibited base

peak at M − 60 and fragment ions at *m/e* 269 [M − (60 + side chain)], 242 [M − (60 + side chain + C-16 + C-17)], and 174 (8). A similar spectrum has been published for the TMSi ether of 3β-hydroxycholest-5-en-7-one (16). These physical properties and the analogous method of synthesis support the structure proposed for 3β,26-diacetoxycholest-5-en-7-one. The reduction to 3β,26-diacetoxy-5α-cholestan-7-one was entirely analogous to that of the 26-deoxy derivative (4). The mass spectrum (Fig. 1) closely resembled that of 3β-acetoxy-5α-cholestan-7-one, with the molecular ion as base peak at *m/e* 502 and a series of fragment ions consonant with the proposed structure as discussed by Budzikiewicz and coworkers (17, 18). Reduction with sodium borohydride of the 7-oxo derivative and hydrolysis provided the 3β,-7,26-triols isomeric at C-7, with the 7α-ol predominating (61% yield). Support for the configuration at C-7 of these isomers is derived from their molecular rotations. The molecular rotation of 5α-cholestane-3β,26-diol ($[\alpha]_D = +27.6°$) is 112°. The contributions of a 7α-ol and 7β-ol are −59° and +110°, respectively (19); thus, the calculated value for 5α-cholestane-3β,7α,26-triol is +53°, and for the 3β,7β,26-triol is +222°. The molecular rotations found for these triols are +53° and +260°, respectively.

With regard to the location of the tritium after catalytic reduction of 3β,26-diacetoxycholest-5-en-7-one, Shapiro et al. (14) and Karliner, Budzikiewicz, and Djerassi (20) have shown that catalytic reduction with deuterium of 3β-acetoxyandrost-5-en-7-one or 3β-acetoxycholest-5-en-7-one provided the 5α,6α-$^2H_2$ derivative, which undergoes loss of 6α-^2H by equilibration with alkali (12). 3β,26-Diacetoxy-5α-[5α,6α-$^3H_2$]-cholestan-7-one lost 31% of the tritium (6α) by similar treatment. Since the 3β,7α,26-triol retained 94% of the tritium of the 7-oxo derivative, 6% of the tritium at C-6 must have been lost during the basic conditions of reduction with sodium borohydride.

This study of the conversion of tritiated 5α-cholestane-3β,26-diol and 3β,7α,26-triol to allo bile acids by the bile fistula rat was initiated to provide comparative data for similar studies with cholestanol (3) and 5α-cholestane-3β,7α-diol (4). An indication of a difference in metabolism between the 3β,26-diol and cholestanol appears in the rate of excretion of labeled biliary acidic metabolites: 26% of administered ^3H in 12 hr from the diol and 11% of injected ^{14}C from cholestanol in 2 days (3). The formation of only 16% allocholic acid from the diol lends support to such a difference. It should be recalled that little (21, 22) or no (23, 24) labeled biliary cholic acid was isolated following administration of labeled 26-hydroxycholesterol to rats. Mitropoulos and Myant (25) have proposed an alternate pathway in bile acid synthesis from cholesterol via 26-hydroxycholesterol to lithocholic acid and thence to its metabolites, 3α,6β-dihydroxy-5β-cholanic acid, chenodeoxycholic acid, and α- and β-muricholic acids. To ascertain whether this pathway is operative with 5α-cholestane-3β, 26-diol, it is necessary to identify the metabolites in fractions associated with monoxy- and dihydroxy bile acids. These studies are in progress.

The biliary acidic metabolites derived from 5α-cholestane-3β,7α,26-triol exhibit a simplified chromatographic pattern (Fig. 4) in comparison with those obtained from 5α-cholestane-3β,26-diol (Fig. 3) or cholestanol (3). Only 1% of the acidic radioactivity appeared in fraction 0-1; 81.6% of the radioactivity in the bile acid fraction was identified as: allochenodeoxycholic acid (28%) and its 3β isomer (4.3%), and allocholic acid (44%) and its 3β isomer (5.3%). These values are similar to those obtained from the metabolism of 5α-cholestane-3β,7α-diol (4) and suggest that these two derivatives are metabolized more rapidly than cholestanol to the 3,7-dihydroxy- and 3,7,12-trihydroxy-5α-cholanic acids. The identification of approximately 50% of the metabolites as trihydroxy-5α-cholanic acids is in marked contrast to the metabolism of cholest-5-ene-3β,7α,26-triol and 5β-cholestane-3α,7α,26-triol, which are converted by the bile fistula rat predominantly into chenodeoxycholic acid and its metabolites, α- and β-muricholic acids (26). The presence of small amounts of di- and trihydroxy-5α-cholestanoic acids as metabolites of 5α-cholestane-3β,7α,26-triol must be considered. A subsequent manuscript will describe the metabolites of this triol resulting from in vitro studies.

The C-26 hydroxyl group in these 5α-cholestane derivatives provided a new asymmetric center at C-25. Since kryptogenin has been converted to diosgenin (27 28), which in turn has the 25R configuration, the materials used in these studies were 25R-5α-cholestane-3β,-7α,26-triol and 25R-5α-cholestane-3β,26-diol, respectively. In the conversion of cholesterol to 5β-cholanic acids, 25R-3α,7α,12α-trihydroxy-5β-cholestanoic acid has been isolated as an intermediate (29–32). The isomeric 25R- and 25S-5β-cholestanoic acids have been prepared and characterized (29, 33–35). Hence, these studies also show that the 25R-5α-cholestane derivatives are converted to the 5α-cholanic acids.

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REFERENCES

1. Karavolas, H. J., W. H. Elliott, S. L. Hsia, E. A. Doisy, Jr., J. T. Matschiner, S. A. Thayer, and E. A. Doisy. 1965. Bile acids. XXII. Allocholic acid, a metabolite of 5α-cholestan-3β-ol in the rat. *J. Biol. Chem.* **240:** 1568–1572.
2. Ziller, S. A., Jr., E. A. Doisy, Jr., and W. H. Elliott. 1968. Bile acids. XXV. Allochenodeoxycholic acid, a metabolite of 5α-cholestan-3β-ol in the hyperthyroid rat. *J. Biol. Chem.* **243:** 5280–5288.
3. Noll, B. W., S. A. Ziller, Jr., E. A. Doisy, Jr., and W. H. Elliott. 1973. Bile acids. XXXVII. Identification of the 3β isomers of allocholic and allochenodeoxycholic acids as metabolites of 5α-cholestanol in the rat. *J. Lipid Res.* **14:** 229–234.
4. Noll, B. W., E. A. Doisy, Jr., and W. H. Elliott. 1973. Bile acids. XXXVIII. Conversion of 5α-cholestane-3β,7α-diol to allo bile acids by the rat. *J. Lipid Res.* **14:** 385–390.
5. Mui, M. M., B. W. Noll, and W. H. Elliott. 1970. 5α-Cholestane-3β,7α,26-triol, an intermediate in the formation of *allo* bile acids from cholestanol. *Federation Proc.* **29:** 909. (Abstr.)
6. Matschiner, J. T., T. A. Mahowald, W. H. Elliott, E. A. Doisy, Jr., S. L. Hsia, and E. A. Doisy. 1957. Bile acids. I. Two new acids from rat bile. *J. Biol. Chem.* **225:** 771–779.
7. Elliott, W. H., L. B. Walsh, M. M. Mui, M. A. Thorne, and C. M. Siegfried. 1969. Bile acids. XXVIII. Gas chromatography of new bile acids and their derivatives. *J. Chromatogr.* **44:** 452–464.
8. Elliott, W. H. 1972. Mass spectra of bile acids. *In* Biochemical Applications of Mass Spectrometry. G. R. Waller, editor. John Wiley, New York. 291–312.
9. Elliott, W. H. 1971. Allo bile acids. *In* The Bile Acids: Chemistry, Physiology, and Metabolism. P. P. Nair and D. Kritchevsky, editors. Plenum, New York, 47–93.
10. Scheer, I., M. J. Thompson, and E. Mosettig. 1956. 5-Cholestene-3β,26-diol. *J. Amer. Chem. Soc.* **78:** 4733–4736.
11. Fieser, L. F. 1953. Cholesterol and companions. II. Exhaustive dichromate oxidation. *J. Amer. Chem. Soc.* **75:** 4386–4394.
12. Beugelmans, R., R. H. Shapiro, L. J. Durham, D. H. Williams, H. Budzikiewicz, and C. Djerassi. 1964. Mass spectrometry in structural and stereochemical problems. LI. Mass spectral and enolization studies on 7-keto-5α-androstanes. *J. Amer. Chem. Soc.* **86:** 2832–2837.
13. Huang-Minlon. 1949. Reduction of steroid ketones and other carbonyl compounds by modified Wolff-Kishner method. *J. Amer. Chem. Soc.* **71:** 3301–3303.
14. Shapiro, R. H., D. H. Williams. H. Budzikiewicz, and C. Djerassi. 1964. Mass spectrometry in structural and stereochemical problems. LIII. Fragmentation and hydrogen transfer reactions of a typical 3-keto steroid, 5α-androstan-3-one. *J. Amer. Chem. Soc.* **86:** 2837–2845.
15. Eneroth, P. 1963. Thin-layer chromatography of bile acids. *J. Lipid Res.* **4:** 11–16.
16. Brooks, C. J. W., E. C. Horning, and J. S. Young. 1968. Characterization of sterols by gas chromatography–mass spectrometry of the trimethylsilyl ethers. *Lipids.* **3:** 391–402.
17. Budzikiewicz, H. 1972. Steroids. *In* Biochemical Applications of Mass Spectrometry. G. R. Waller, editor. Wiley-Interscience, New York. 251–289.
18. Budzikiewicz, H., C. Djerassi, and D. H. Williams. 1964. Structure Elucidation of Natural Products by Mass Spectrometry. Vol. II: Steroids, Terpenoids, Sugars, and Miscellaneous Classes. Holden-Day, San Francisco. 79–81.
19. Klyne, W. 1960. The Chemistry of the Steroids. Methuen, London, 55.
20. Karliner, J., H. Budzikiewicz, and C. Djerassi. 1966. Mass spectrometry in structural and stereochemical problems. XCI. The electron impact induced elimination of water from 3-hydroxy steroids. *J. Org. Chem.* **31:** 710–713.
21. Wachtel, N., S. Emerman, and N. B. Javitt. 1968. Metabolism of cholest-5-ene-3β,26-diol in the rat and hamster. *J. Biol. Chem.* **243:** 5207–5212.
22. Javitt, N. B., and S. Emerman. 1970. Metabolic pathways of bile acid formation in the rat. *Mt. Sinai J. Med.* **37:** 477–481.
23. Danielsson, H. 1961. Formation and metabolism of 26-hydroxycholesterol. *Ark. Kemi.* **17:** 373–379.
24. Fredrickson, D. S., and K. Ono. 1956. The *in vitro* production of 25- and 26-hydroxycholesterol and their *in vivo* metabolism. *Biochim. Biophys. Acta.* **22:** 183–184.
25. Mitropoulos, K. A., and N. B. Myant. 1967. The formation of lithocholic acid, chenodeoxycholic acid and α- and β-muricholic acids from cholesterol incubated with rat-liver mitochondria. *Biochem. J.* **103:** 472–479.
26. Berséus, O., and H. Danielsson. 1963. On the metabolism of coprostane-3α,7α-diol in mouse liver homogenates. *Acta Chem. Scand.* **17:** 1293–1298.
27. Kaufmann, S., and G. Rosenkranz. 1948. Steroidal sapogenins. I. Transformation of kryptogenin into diosgenin and pseudodiosgenin. *J. Amer. Chem. Soc.* **70:** 3502–3505.
28. Varma, K. R., J. A. F. Wickramasinghe, and E. Caspi. 1969. Biosynthesis of plant sterols. IX. The mode of oxygenation at carbon atom 26 in the formation of sapogenins from cholesterol. *J. Biol. Chem.* **244:** 3951–3957.
29. Staple, E., and J. L. Rabinowitz. 1962. Formation of trihydroxycoprostanic acid from cholesterol in man. *Biochim. Biophys. Acta.* **59:** 735–736.
30. Carey, J. B., Jr., and G. A. D. Haslewood. 1963. Crystallization of trihydroxycoprostanic acid from human bile. *J. Biol. Chem.* **238:** 855PC–856PC.
31. Carey, J. B., Jr. 1964. Conversion of cholesterol to trihydroxycoprostanic acid and cholic acid in man. *J. Clin. Invest.* **43:** 1443–1448.
32. Mendelsohn, D., and L. Mendelsohn. 1969. The catabolism of cholesterol *in vitro*. Formation of 3α,7α,12α-trihydroxy-5β-cholestanoic acid from cholesterol by rat liver. *Biochem. J.* **114:** 1–3.
33. Bridgwater, R. J. 1956. Partial synthesis of the two 3α,-7α,12α-trihydroxycoprostanic acids and of similar bile acids with extended side chains. *Biochem. J.* **64:** 593–599.
34. Briggs, T. 1970. Partial synthesis of 25_D- and 25_L-cholestanoic acids from some common bile acids. *J. Org. Chem.* **35:** 1431–1434.
35. Hoshita, T., and T. Kazuno. 1968. Chemistry and metabolism of bile alcohols and higher bile acids. *Adv. Lipid Res.* **6:** 207–254.