Evidence for Bile Acid Synthesis by Transplantable Hepatomas

Glen E. Mott, 2 Henry C. Pitot, and Stanley Goldfarb

SUMMARY

Transplantable Morris hepatomas 5123C and 9618A were assayed for enzymes known to be active in the biosynthesis of bile acids in liver. Cholesterol 7α-hydroxylase, the initial enzyme in the pathway of bile acid biosynthesis, was shown to be as active in 9618A tumor microsomes as in host liver microsomes. Activity of this enzyme in the 5123C tumor was about one-third that of host liver. 7α-Hydroxycholesterol was shown to be metabolized by tumor microsomes to other probable intermediates. Cleavage of the sterol side chain, a part of the hepatic bile acid metabolic pathway, was also detected in both 9618A and 5123C tumor mitochondrial preparations. Labeled chenodeoxycholic acid was identified in extracts from tumor and liver slices that were incubated in the presence of sodium mevalonate-3H.

INTRODUCTION

Considerable effort has been made in recent years to elucidate the regulatory mechanisms of cholesterol biosynthesis by tumors. These efforts have resulted largely from the observations of Siperstein (33) with Fagan (34) that cholesterol synthesis by hepatomas is not inhibited by cholesterol feeding as has previously been shown for liver (36). As the principal end products of cholesterol metabolism in liver, bile acids have a regulatory effect on intestinal cholesterol synthesis (9), bile acid synthesis (31), and possibly hepatic choledochogenesis (14). However, evidence of bile acid synthesis in hepatomas had been lacking, although an indication of bile casts in several tumors was noted by Reuber (28). The present work was initiated to determine whether bile acids are synthesized by Morris transplantable hepatomas 5123C and 9618A and to compare the relative activity of the initial enzyme, cholesterol 7α-hydroxylase, in bile acid synthesis, in the transplantable tumors and in host liver.

MATERIALS AND METHODS

Morris 5123C and 9618A hepatomas were transplanted by injection of whole-cell suspensions into the thighs of Buffalo rats. Generation time for the 5123C was 6 to 8 weeks and 13 months for the very highly differentiated 9618A tumor.

Animal Procedures. Tumor-bearing male rats weighing 150 to 300 g were fed a laboratory chow ration (Wayne Lab Blox; Allied Mills, Inc., Chicago, Ill.) containing 2% cholestyramine plus 2% corn oil, a diet shown to stimulate cholesterol synthesis (12) and bile acid production. The animals were fed ad libitum on a reverse day-night light cycle, i.e., lights on from 8:00 p.m. to 8:00 a.m. and off during the day for at least 10 days before sacrifice. The animals were decapitated at noon and the tumors and host livers were removed and placed on ice.

Bile Acids in Tumors. An attempt was made to isolate bile acids from the Morris 9618A and 5123C tumors. About 0.5 g of lyophilized tissue was extracted and saponified according to the method of Evrard and Janssen (10). The bile acid fraction was methylated with diazomethane and trifluoroacetylated with trifluoroacetic anhydride (25). The residue was taken up in CS2 and analyzed by GLC.

Cholesterol 7α-Hydroxylase Assay. The initial and rate controlling step in the hepatic bile acid pathway, the conversion of cholesterol to 7α-hydroxycholesterol, was assayed by a technique similar to that of Mitton et al. (23). Host livers and tumors from 3 rats from each tumor group were homogenized in 3 volumes of 154 mM KCl and 1 mM EDTA, pH 7.4, with 4 strokes of a Teflon:glass Potter-Elvehjem homogenizer and centrifuged at 18,000 × g for 15 min in a refrigerated centrifuge. The supernatant was carefully removed and centrifuged at 100,000 × g for 1 hr. The microsomal pellet was resuspended in the KCl/EDTA medium to give a suspension of 1 to 2 mg microsomal protein per 0.1 ml. Protein was determined according to the method of Lowry et al. (21).

The incubation mixture, which contained microsomal protein, 100,000 × g supernatant, and the necessary cofactors, is given in Table 1. Addition of the 100,000 × g supernatant stimulated the enzyme activity 70 to 90%. The supernatant used in the 7α-hydroxylase assay of the liver

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2 NIH Postdoctoral Fellow, Grant 1-F2-CA-52, 719-01. Present address: Departments of Pathology and Biochemistry, The University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, Texas 78284.
3 The abbreviations used are: GLC, gas-liquid chromatography; TLC, thin-layer chromatography; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A. The trivial names used are: cholesterol, cholest-5-ene-3β-ol; 7α-hydroxycholesterol, cholest-5-ene-3β,7α-diol; 7β-hydroxycholesterol, cholest-5-ene-3β,7β-diol; 7-ketocholesterol, cholest-5-ene-3β-ol-7-one; 7α-hydroxy-4-cholesten-3-one, cholest-4-ene-7α-ol-3-one.
4 A preliminary report was presented at the Federation of American Societies for Experimental Biology, April 1973 (24).
was pooled from liver preparations. A similar pooled supernatant from tumor preparations was used in the assay of tumor microsomes. No differences were observed in the stimulation by 100,000 g supernatant from tumor or liver. Radioactive cholesterol in 10 µl of acetone (59.8 mCi/mmole, New England Nuclear, Boston, Mass.) was mixed with the microsomal cholesterol that served as substrate pool, approximately 40 nmole cholesterol per mg microsomal protein.

Incubation time was 30 min at 37° with shaking. The reaction was stopped by 3 extractions with 3 ml of diethyl ether. The ether solution was evaporated to dryness, the residue was dissolved in chloroform, and this solution was spotted on a thin layer of Silica Gel G with 7α-hydroxycholesterol and 7β-hydroxycholesterol and cholesterol standards. The plate was developed in ether, sprayed with 0.01% dichlorofluorescein in methanol, visualized under UV light, and the bands were scraped into scintillation vials containing 10 ml of scintillation solvent.

**Total Cholesterol Determination.** Liver and 5123C tumor microsomes from 6 rats were saponified with ethanolic KOH and extracted with hexane (1). Tridecanoin, which was standardized against a pure cholesterol sample, was dissolved in chloroform and added to the hexane extract as an internal standard for GLC analysis (19). An aliquot of the mixture was injected in chloroform on a glass column (0.25 inch x 3 feet) packed with 3% OV-17 on 100 to 120 mesh Gas Chrom Q (Applied Science Laboratories, State College, Pa.) at 300°. Helium gas flow was 75 ml/min. Peak areas were determined by triangulation and cholesterol concentrations were calculated.

**7α-Hydroxycholesterol Dehydrogenase.** Metabolism of 7α-hydroxycholesterol was also determined using microsomal protein, 100,000 g supernatant, and the necessary cofactors as shown in Table 1. 7α-Hydroxycholesterol-7β-3H (51 mCi/mmole), 275,000 dpm, was used as substrate was synthesized from 7-ketocholesterol and NaB3H4 (102 mCi/mmole, New England Nuclear) (7). The assay mixture was incubated at 37° for 30 min and stopped by extraction with ether. The products were separated by TLC with ether as solvent, and the radioactivity was counted as in the 7α-hydroxycholesterol assay. Several other more polar metabolites were produced if an NADPH-generating system was used in addition to NAD+. Side-Chain Cleavage. Since the final steps in the bile acid pathway require oxidative cleavage of the neutral sterol side chain, metabolism of the resulting 3-carbon fragment produces CO2. Thus, liberation of labeled CO2 from the sterol side-chain has been used as a measure of side-chain cleavage activity (22, 39). Mitochondrial preparations were made from host liver, the 2 types of tumors, kidney, and brain by the procedures of Whitehouse et al. (39). Heart mitochondria were prepared by the procedure of Crane (13). The mitochondria were washed in 10% sucrose at least twice and incubated in 25-ml Erlenmeyer flasks with the incubation mixture of Mitropoulos and Myant (22) except without the soluble supernatant factor. The substrate, 1 x 106 dpm of cholesterol-26-C14 (61 mCi/mmole; Amersham-Searle Corp., Arlington Heights, Ill.) was dissolved in warm buffer, Tween 80 (5 µl), and a trace of acetone. Optimum activity was obtained by the addition of about 5 mg microsomal protein to the mitochondrial preparations, although electron microscopy of the “purified” mitochondrial preparations also indicated some microsomal contamination. The flasks were sealed with serum stoppers fitted with a suspended plastic center well, and incubated at 37° for 8 hr in a rotary shaker. The reaction was stopped with 2 ml of 6 N HCl, and 0.3 ml of ethanolamine/methyl cellosolve (1/1) was added to the center well to trap CO2. Shaking was continued for another 30 min, and the flasks were placed in a 37° oven for 4 hr. The contents of the center well were counted by liquid scintillation in Scintisol.

**Bile Acid Synthesis by Tissue Slices.** Approximately 500 mg of slices were made from host liver, 9618A tumors, and 5123C tumors with a Stadie-Riggs tissue slicer. The slices were incubated for 3 hr at 37° in the presence of 5 ml of Krebs-Ringer-phosphate buffer, pH 7.4 (38) and 5 µCi (2 µmoles) of sodium mevalonate-5-3H (New England Nuclear). The assay mixture was saponified in 1.25 N KOH for 3 hr at 15 psi and the neutral steroids were extracted 5 times with 5 ml of hexane. Hexane extracts were fractionated by development of TLC plates of Silica Gel G in hexane/diethyl ether/acetone spray reagent. The band corresponding to the chenodeoxycholic acid standard was eluted with chloroform/methanol (2/1), and the solvent was evaporated. The residue was...
treated with alcoholic diazomethane in ether, and the trifluoroacetate derivatives were prepared as described previously (25). The residue was taken up in chloroform and injected on a glass column (6 feet × 0.25 inch) packed with 3% OV-17 (Supelco, Inc., Bellefonte, Pa.). The effluent was diverted into the ionizing chamber of a Finnegan Model 1015C mass spectrometer combined with a Finnegan System 150 for data output.

RESULTS

7α-Hydroxylase Assay

Significant cholesterol 7α-hydroxylase activity was found in both the 9618A and 5123C tumors and the host livers as shown in Table 1. The activity is expressed as percentage of total radioactivity converted to 7α-hydroxycholesterol per 30 min incubation per 0.5 mg microsomal protein. Preliminary studies indicated that added cholesterol does not completely mix with the microsomal cholesterol so that the actual substrate pool size cannot be accurately determined. A recent report verifies this observation (2) and gives evidence that the enzyme cannot be completely saturated with respect to substrate. In the same report it was demonstrated that addition of as much as 300 nmoles of exogenous cholesterol per assay increased the effective substrate pool size less than 10%. Thus, calculation of 7α-hydroxylase activity based on radioactivity of the product and specific radioactivity of the substrate would lead to large errors in estimation of enzyme activity.

The assay used in the present work is similar to that reported by Boyd et al. (3) in which the 7α-hydroxylase activity, expressed as percentage of total radioactive cholesterol converted to 7α-hydroxycholesterol, increases dramatically as does bile acid output with bile duct cannulation or cholestyramine feeding. In the present experiment, the assay is linear up to about 30 min. The cholesterol contents of the microsomes from the tumors and host liver are very similar. Total cholesterol determinations gave 15.9 ± 1.97 (mean ± S.E.) µg cholesterol per mg microsomal protein from liver and 16.0 ± 0.94 µg/mg for the 5123C tumor. Therefore, the enzyme activity is represented by the percentage of cholesterol-4-14C converted to 7α-hydroxycholesterol.

With these considerations in mind the 7α-hydroxylase activity of the very highly differentiated 9618A is comparable to that of host liver, but that of the 5123C is considerably lower. No 7α-hydroxylase activity was found in microsomal preparations from kidney, brain, or heart. In other experiments the 7α-hydroxylase activity was always 25 to 30% lower in the liver of hepatoma-bearing rats than in the livers of normal rats without tumors. This could result from inhibition of liver bile acid synthesis by bile acids produced and released by the transplantable hepatoma.

7α-Hydroxycholesterol Dehydrogenase

The conversion of 7α-hydroxycholesterol to 7α-hydroxy4-cholesten-3-one, the 2nd step in hepatic bile acid synthesis, proceeded readily in the presence of NAD+ and microsomes from host livers and tumors as shown in Table 1. Conversion of 7α-hydroxy-4-cholesten-3-one to more polar intermediates in the pathway was stimulated by the addition of an NADPH-generating system to the above preparations as shown in Table 2, Column 3. The TLC mobilities of these intermediates from representative incubations of host liver and 5123C tumor microsomes were consistent with those previously reported (8) while compounds migrating near the solvent front were not identified. The same intermediates appeared to be formed in both tumors and host liver preparations although no further attempt was made at identification.

Again the very highly differentiated 9618A tumor microsomes were more active in metabolism of 7α-hydroxycholesterol than the liver or the 5123C preparations. Although there is no adequate explanation for the lower activity of cholesterol 7α-hydroxylase or 7α-hydroxycholesterol dehydrogenase in the 5123C tumor preparations, it has also been shown that the HMG-CoA reductase activity (EC 1.1.1.34) of the 5123C tumor is about one-third that of the 9618A tumor (11).

Side-Chain Cleavage

Side-chain cleavage as measured by 14CO2 production from mitochondrial metabolism of cholesterol-26,14C was demonstrated in host liver and 9618A and 5123C hepatoma mitochondrial preparations. Table 3 shows the cpm of 14CO2 trapped from the various incubations. No side-chain cleavage was noted by preparations from kidney, brain, or heart. Liver and tumor mitochondrial preparations showed enhanced activity if microsomes were added, which was further stimulated by the addition of NADPH. However, liver and especially tumor mitochondrial preparations gave activity without added microsomes. This activity may be partially attributed to microsomal contamination which was always found in the mitochondrial preparations as shown by electron microscopy. The actual substance in the in vitro assay and the localization of some of the enzymes involved in side-chain cleavage is still in doubt (6, 27). However, many enzymes functioning in the early steps of the bile acid pathway are microsomal, which could account for the stimulation of side-chain cleavage.

Bile Acids

Whole-Tumor Extracts. Bile acids could be detected only in trace amounts in extracts from liver and 9618A and 5123C tumors when analyzed by GLC as methyl ester trifluoroacetates. Small amounts of material with the same retention times as chenodeoxycholic and cholic acids were detected as were several other unidentified compounds.

Tissue Slices. TLC of the extracted neutral sterols from liver and hepatoma slices gave an average of 25,000 dpm in the cholesterol and cholesterol ester bands. TLC of the tumor and liver slice ether extracts gave 3,920 and 3,815 dpm, respectively, and the 5123C extracts less than 1000 dpm in the bands corresponding to chenodeoxycholic acid. Aliquots were evaporated, derivatized, and analyzed by
Table 2
Microsomal metabolites of 7α-hydroxycholesterol-7β-3H (TLC separation)

<table>
<thead>
<tr>
<th>Total dpm recovered/30 min/0.5 mg microsomal protein</th>
<th>1. -NAD, -NADPH</th>
<th>2. +NAD, -NADPH</th>
<th>3. +NAD, +NADPH</th>
<th>4. -NAD, +NADPH</th>
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</thead>
<tbody>
<tr>
<td>Host liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solvent front</td>
<td>376</td>
<td>1,424</td>
<td>891</td>
<td>1,103</td>
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<tr>
<td></td>
<td>235</td>
<td>1,434</td>
<td>609</td>
<td>436</td>
</tr>
<tr>
<td></td>
<td>361</td>
<td>5,778</td>
<td>1,673</td>
<td>158</td>
</tr>
<tr>
<td>7αHCEO*</td>
<td>764</td>
<td>63,500</td>
<td>12,261</td>
<td>3,412</td>
</tr>
<tr>
<td>7βHC</td>
<td>5,482</td>
<td>4,062</td>
<td>4,701</td>
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<tr>
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<td>5,570</td>
<td>11,012</td>
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<td>3,152</td>
<td>15,226</td>
<td>12,036</td>
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<td>5123C tumor</td>
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<td></td>
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<td>Solvent front</td>
<td>155</td>
<td>463</td>
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<td></td>
<td>425</td>
<td>1,202</td>
<td>671</td>
<td>692</td>
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<td>613</td>
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<td>3,776</td>
<td>4,720</td>
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<tr>
<td>7αHC</td>
<td>233,740</td>
<td>219,656</td>
<td>200,261</td>
<td>236,347</td>
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<td>9,706</td>
<td>6,257</td>
<td>17,472</td>
<td>8,589</td>
</tr>
<tr>
<td>Origin</td>
<td>2,749</td>
<td>1,545</td>
<td>10,262</td>
<td>12,661</td>
</tr>
</tbody>
</table>

* 7αHCEO, 7α-hydroxy-4-cholesten-3-one; 7βHC, 7β-hydroxycholesterol; 7αHC, 7α-hydroxycholesterol.

Table 3
14CO₂ produced from mitochondrial side-chain cleavage of cholesterol-26-14C

<table>
<thead>
<tr>
<th>Total radioactivity (dpm 14CO₂)</th>
<th>9618A tumor</th>
<th>9618A host liver</th>
<th>5123C tumor</th>
<th>5123C host liver</th>
<th>Heart</th>
<th>Kidney</th>
<th>Brain</th>
</tr>
</thead>
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<tr>
<td>Mitochondria*</td>
<td>14,724</td>
<td>150</td>
<td>3,100</td>
<td>1,100</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>+ Microsomes*</td>
<td>44,897</td>
<td>25,700</td>
<td>10,096</td>
<td>4,871</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Assay mix: 20 mg mitochondrial protein; 0.1 M Tris-HCl buffer, pH 8.5; 2.3 mM ATP; 0.4 mM NAD; 3.0 mM glutathione; 2.2 mM MgCl₂; 4.0 mM sodium citrate; 1 x 10⁶ dpm cholesterol-26-14C per assay; 1 mg streptomycin per assay; 2000 IU penicillin G per assay.

** Five mg microsomal protein; 0.5 mM NAD⁺; 1.0 mM glucose-6-P; 15 IU glucose-6-P dehydrogenase per assay.

GLC. Mass spectra of the GLC effluent at the same retention time as a pure methylchenodeoxycholate dimethylfluoroacetate standard gave approximately the same ratio of peak intensities at m/e 225, 369, and 484. Peaks of material present. These data indicate a trace of chenodeoxycholate present in the tumor slices. We were unable to detect cholic acid using the same procedure.

The work of Kellogg and Wostmann (18) indicates that chenodeoxycholic acid is a major bile acid in germ-free rat bile, which confirms a previous report that chenodeoxycholic acid is a primary bile acid in the rat (15), although cholic acid and muricholic acids predominate. In the latter study it was also demonstrated that labeled taurochenodeoxycholic acid is synthesized by liver within 1 to 2 hr after perfusion with cholesterol-4-14C, while cholic acid conjugates did not appear for 2 to 3 hr. This may explain the synthesis of chenodeoxycholic acid by the tumor slices as the principal bile acid and the absence of cholic acid.

DISCUSSION

In this study we have shown that the Morris 5123C hepatoma and the much slower growing and better differentiated Morris 9618A hepatoma can carry out 3 of the same partial synthetic reactions for bile acid production (24) previously demonstrated in normal liver. These include 2 initial microsomal enzymatic reactions and the terminal cleavage of the sterol side chain by mitochondria. The 1st reaction, mediated by cholesterol 7α-hydroxylase, is rate controlling for the entire bile acid-biosynthetic pathway in normal rat liver (32). Although bile acids were identified in
extracts of the tumors, there was no appreciable accumulation of bile acids in the tissues.

Since hepatic bile acid and cholesterol biosynthesis are closely integrated (40), it is especially interesting that in the 9618A hepatoma both the cholesterol 7α-hydroxylase and the HMG-CoA reductase activities are 3 times that found in the 5123C tumor. HMG-CoA reductase is known to be rate controlling for cholesterol synthesis in the normal liver (20) and several experiments suggest that the enzyme is rate limiting in transplantable hepatomas. First, the most highly differentiated hepatomas incorporate much more labeled acetate into cholesterol (29) and also have proportionally higher levels of microsomal HMG-CoA reductase (11) than the less differentiated hepatomas. Secondly, the 5123C but not the 9618A hepatoma shows a diurnal rhythm of HMG-CoA reductase activity (11) that is parallel to the presence and absence, respectively, of the in vivo cyclic synthesis of cholesterol (30). By analogy with cholesterol synthesis, bile acid production is possibly regulated by cholesterol 7α-hydroxylase in the hepatomas. If it is rate limiting, the high activity of this enzyme in the tumors suggests a considerable synthesis of bile acids.

There appears to be a specific microsomal cholesterol pool which serves as substrate for bile acid synthesis in rat liver (2). Kinetic studies indicate (35) that cholesterol for bile acid synthesis is derived from a fraction that is first transferred to the blood and only later reenters the liver for further oxidation to bile acids. There is indirect evidence that over 90% of the cholesterol produced by hepatomas is released into the blood circulation (5, 26). These observations may indicate that in spite of a slower rate of uptake of dietary cholesterol by hepatomas than by liver (16, 17) the cholesterol substrate pool for hepatoma bile acid synthesis may be derived from plasma rather than endogenous synthesis.

ACKNOWLEDGMENTS

We wish to thank Dr. Thomas Barber, Department of Pathology, University of Wisconsin, for electron microscopy of the mitochondrial preparations and Susan Weintraub, University of Texas Health Science Center at San Antonio, for the mass spectral analyses.

ADDENDUM

After this paper was submitted, another report appeared (4) that also presented evidence for bile acid synthesis by hepatomas.

REFERENCES

Bile Acid Synthesis by Transplantable Hepatomas


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