Effects of Dietary Cholesterol on Bile Acid Synthesis in Liver and Hepatomas

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SUMMARY

The effects of cholesterol feeding on bile acid production in vitro in liver were contrasted with those in a strain of Morris hepatoma active in sterol synthesis. The principal conclusions are as follows:

Bile acid production de novo from acetate in liver is suppressible by dietary cholesterol in a fashion exactly comparable with, and related to, the cholesterol negative feedback phenomenon.

In liver, a dual, reciprocating precursor supply is available for bile acid formation. Dietary cholesterol, when present, functions in the presence of blocked sterol synthesis, as the sole precursor of bile acid.

In the presence of a high-cholesterol diet, liver has the ability to convert mevalonate and stored dietary cholesterol simultaneously to bile acid.

Bile acid synthesis by a malignant tumor is described in this report. The tumor (Morris hepatoma 3924A) is active in bile acid production from both acetate and mevalonate, but these syntheses are totally unaffected by dietary cholesterol. Moreover, dietary cholesterol cannot itself be utilized by the tumor for bile acid synthesis.

INTRODUCTION

Hepatomas of all degrees of differentiation have served as biochemical models of altered hepatic biochemistry in many laboratories (8). Of interest to us in recent years have been the descriptions of a number of defects in regulation of lipogenesis in these lesions, particularly involving the biosynthesis of cholesterol (11). Investigations of these tumors in vivo have demonstrated that sterols produced by hepatomas actually circulate in lipoproteins in the peripheral blood and form the basis of a theoretically useful means of detecting the presence of hepatomas (3).

The present studies seek to expand the data concerning sterol synthesis in hepatomas a step beyond sterols themselves to their major metabolic end products, the bile acids. Using Morris hepatoma 3924A, an undifferentiated adenocarcinoma of liver with active capacity for sterol synthesis, we have demonstrated that several molecular species of bile acids are synthesized. This represents, to our knowledge, the 1st example of bile acid synthesis found in a malignant tumor.

MATERIALS AND METHODS

Animals and Tumors. As mentioned above, Morris hepatoma 3924A was used exclusively in these studies. A number of healthy, tumor-free rats were maintained on the same diets and served as liver donors in control studies. The tumors, transplanted in the right thighs of recipient ACF/Mai rats, had reached weights of approximately 5 g. Host animal weights were 150 to 250 g. Animals were kept on 1 of 2 diets, either a normal Purina rat chow, "low-cholesterol" diet, or a "high-cholesterol" diet, consisting of 5% cholesterol and 10% oleic acid, to which sufficient cholesterol-1α,2α-3H had been added to give the cholesterol a specific radioactivity of 3750 (±5%) dpm/μmole. In the latter case, the high-cholesterol-3H diet was maintained for 48 to 72 hr before the animal was used in the experiment. Nonradioactive cholesterol (Nutritional Biochemicals, Inc., Chagrin Falls, Ohio) used in the diet was recrystallized 3 times in ethanol.

At the time of each experiment the animal was stunned by a blow on the head, and its tumor was grossly dissected out of its position in its capsule in the thigh muscles. In the tumor-free animals, the liver was quickly perfused with 20 ml of iced Krebs bicarbonate buffer via the portal vein and removed. Tumors and livers, treated separately, were sliced by hand into ribbons of tissue and placed on a Mickle tissue slicer. Slices were 0.33 mm thick. Either 500 mg of tumor tissue or 350 mg of normal liver were used per flask.

Tissue Incubation. All incubations were carried out in the outer portions of 25-ml center well flasks. In addition to the tissue slices were 2 ml of Krebs bicarbonate buffer, pH 7.40, and a labeled precursor of bile acids, either sodium acetate-2-14C or mevalonolactone-2-14C. In the former case, 2 μCi (specific radioactivity, 1.44 μCi/μmole) were added; and in the latter, 2 μCi (specific radioactivity, 12.9 μCi/μmole) were added to the mixture. The contents of the flask were gassed with a mixture of 95% O2:5% CO2, capped, and incubated for 2 hr in a Dubnoff shaker at 38° and 100 oscillations/min, as described elsewhere (2).

Extractions. At the end of the incubation the reactions were stopped by the injections through the stoppers of 0.5 ml of Hyamine hydroxide into the center well and 0.25 ml of
The samples were then transferred to 50-ml screw cap tubes, extractable by this procedure. The alkaline mixture was then autoclaved at 15 psi for 2 hr (at 250°) to deconjugate extraction tubes, along with 0.5 ml of 90% KOH. In excess 40 ml of petroleum ether to remove nonsaponifiable lipids, absolute ethanol. The combined washes were added to the and the original flasks were washed 3 times with 2 ml of pH was lowered to 1.0 to 2.0 with concentrated HC1 and including digitonin-precipitable sterols. Following this, the extraction step was repeated a 2nd time, and the extracts were combined in the beaker. The ether extract, containing the deconjugated bile acids, was allowed to dry. It was then transferred to a 50-ml screw cap tube by washing the beaker with 2 successive washes of 2 ml of methanol.

Methyl esters of bile acids were made by a modification of the method of Mosbach et al. (9). Specifically, 4 drops of concentrated HCl were added to the methanol solution and allowed to remain overnight. The following day 8 ml of benzene:ether (3:2) and 4 ml of H2O were added to each tube. Two phases developed, with the bile acid methyl esters extracting almost totally into the upper phase on vortexing. Two repeat extractions with benzene:ether were combined with the first, each being carefully removed with a Pasteur pipet and placed in a 100-ml beaker. This combined extract was allowed to dry under a gentle stream of nitrogen. A small quantity (0.5 g) of dried diatomaceous earth was moistened with 0.4 ml of equilibrated glacial acetic acid (see below) and added to the beaker to absorb the methyl esters. This procedure was repeated, and the Celite-bile acid methyl ester complex, thoroughly mixed in the beaker, was applied to the top of the column.

**Column Chromatography.** Glass columns were prepared according to a modification of the method of Matschner et al (6). Columns were 45 cm long, 15 mm in diameter, and filled with 8.3 g of equilibrated Celite. Celite was prepared by repeated washing in methanol, gentle oven drying, and then mixing with 6.4 ml acetic acid:H2O (7:3, v/v) which had been previously equilibrated with, and then separated from, hexane (40 volumes). This treated Celite mixture was then firmly packed into the column atop a porcelain plate with a stopcock below. The bile acid methyl ester-Celite complex was then added to the column and packed.

Known standards of bile acids were saponified, extracted, and methylated; the methyl esters of the bile acids on this column eluted such that those of monohydroxylated bile acids appeared in the 100% hexane fraction. Those of chenodeoxycholate and its congeners appeared in the 80% hexane:20% benzene fraction. Methyl chololate and its congeners appeared in the 40% hexane:60% benzene fraction. Between those fractions, in the 60% hexane:40% benzene elution appeared the methyl muricholates. Products appearing in the 20% and 0% hexane fractions were unidentified.

**Reverse Isotope Dilution.** Substantiation of bile acid production was achieved by isolating an aliquot of the 40% hexane, and of the 80% hexane, fractions from extract of 3924A hepatoma slices incubated with mevalonate-2-14C. To the former were added 200 mg of methyl cholate (prepared from authentic cholic acid); and to the latter were added 200 mg of methyl chenodeoxycholate (prepared from authentic chenodeoxycholic acid). Bile acids were methylated as described above. Each 200-mg sample was divided into 3 equal parts (Fractions 1, 2, and 3); each Fraction 1 was dissolved in methanol in centrifuge tubes and recrystallized by dropwise addition of water, followed by a period of several hr to allow for crystal development. These were then harvested by centrifugation, with removal of the supernatant; the crystals were then dried, weighed, and counted. Fractions 2 and 3 were treated identically except that, in the case of these fractions, the crystals were redissolved and recrystallized once and twice more, respectively, prior to weighing and counting.

**Gas-Liquid Chromatography.** Representative samples collected from the columns above were nitrogen dried and taken up in a few µl of methanol and injected onto a 6-foot QF-1 column in a Packard 823 gas-liquid chromatograph, using an argon ionization detection system. Gas flow was at 90 ml/min. Inlet temperature was 275°, column temperature was 265°, and detector temperature was 270°.

**Liquid Scintillation Counting.** Fractions from column chromatography were dried down under nitrogen. Each fraction was taken up in 1.0 ml of methanol and transferred to a scintillation vial to which were added 15 ml of a toluene-based scintillation fluid constructed as described elsewhere (4).

A Packard 3390 liquid scintillation counter was the instrument used. The machine was equipped with an Absolute Activity Analyzer which, with appropriate quench curves and internal standardization, assisted in the determination of dpm in 3H and 14C channels.

**RESULTS**

**Effects of Dietary Cholesterol on Bile Acid Synthesis in Liver.** Table 1 shows the appearance of eluted radioactivity in bile acid methyl esters in the changing solvent system. This table, showing the in vitro conversion of acetate-2-14C to bile acid, demonstrates the effects of a high-cholesterol diet. Pretreatment with the high-cholesterol diet considerably suppressed the ability of the liver to convert acetate to bile acid, as expected. Moreover, this "suppressed" liver from the cholesterol-fed animal converted dietary sterol (instead of externally added acetate) to bile acid. Clearly, the liver can utilize de novo sterol as a precursor for its bile acid, or, if this sterol production is inhibited by dietary cholesterol via the cholesterol negative feedback phenomenon, the dietary cholesterol can itself be used as a bile acid precursor with apparently equal facility.
Table 1

**Effects of dietary cholesterol-3H on the incorporation of acetate-2-14C and mevalonate-2-14C to bile acid in liver**

Radioactivity in eluates of bile acid methyl esters from Celite columns is presented. Each of the hexane: benzene fractions shown was in a volume of 100 ml, eluted from the column in 4 successive 24-ml aliquots. Numbers tabulated are the integrated values of the 4 aliquots in each fraction.

<table>
<thead>
<tr>
<th>Fraction (% hexane: % benzene)</th>
<th>Low-cholesterol diet (acetate to bile acid)</th>
<th>High-cholesterol diet (acetate to bile acid)</th>
<th>Low-cholesterol diet (mevalonate to bile acid)</th>
<th>High-cholesterol diet (mevalonate to bile acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100:0</td>
<td>3400</td>
<td>820</td>
<td>0</td>
<td>2130</td>
</tr>
<tr>
<td>80:20</td>
<td>1170</td>
<td>300</td>
<td>0</td>
<td>1690</td>
</tr>
<tr>
<td>60:40</td>
<td>3540</td>
<td>370</td>
<td>220</td>
<td>2670</td>
</tr>
<tr>
<td>40:60</td>
<td>6620</td>
<td>470</td>
<td>310</td>
<td>3570</td>
</tr>
<tr>
<td>20:80</td>
<td>5740</td>
<td>340</td>
<td>290</td>
<td>2630</td>
</tr>
<tr>
<td>0:100</td>
<td>2870</td>
<td>220</td>
<td>60</td>
<td>1940</td>
</tr>
</tbody>
</table>

This highly malignant, undifferentiated adenocarcinoma of liver incorporates acetate into bile acid, and, as shown in the table, in amounts comparable to that of liver. The lack of suppressive effect of dietary cholesterol on this synthetic process in the hepatoma is demonstrated. This tissue, moreover, was essentially unable to use dietary cholesterol as a precursor for synthesis of its own bile acid, despite its easy accessibility in the circulation in vivo (Table 3).

This latter observation, the failure of the neoplastic tissue to utilize dietary cholesterol as a bile acid precursor, was examined in relation to the innate abilities of liver and hepatoma 3924A to store dietary cholesterol. Results for these tissues on the relative quantities of free and total digitonin-precipitable sterol after 2 hr of incubation with acetate-14C are contrasted in Table 3. The hepatoma is clearly unable to store cholesterol derived from diet, an observation closely related to the data shown in Table 2.

The fractions in the 80% hexane: 20% benzene elution of methyl esters generally contain dihydroxy bile acids, as mentioned. The eluate of the first of the 4 portions of this fraction from a representative sample were subjected to gas-liquid chromatography together with a known standard of methyl chenodeoxycholate eluted from a similar column. Peaks in the eluate occurred at a retention time of 8 min, as did the peak representing the standard. Such chromatograms strongly suggest that one of the peaks in the sample

Table 3

**Storage of dietary cholesterol-3H and bile acid in liver and in Morris hepatoma 3924A**

<table>
<thead>
<tr>
<th>Rat</th>
<th>Cholesterol (mg/100 ml)</th>
<th>Cholesterol-3H (dpm/ml)</th>
<th>Sterol (mg/g)</th>
<th>dpm 3H in cholesterol/g tissue</th>
<th>dpm 3H in bile acid/g tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total</td>
<td>Free</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>203</td>
<td>16,900</td>
<td>31.0</td>
<td>4.5</td>
<td>77,300</td>
</tr>
<tr>
<td>2</td>
<td>180</td>
<td>15,000</td>
<td>39.0</td>
<td>5.0</td>
<td>90,900</td>
</tr>
<tr>
<td></td>
<td><strong>Normal animal</strong>—liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>317</td>
<td>15,000</td>
<td>2.0</td>
<td>1.5</td>
<td>1,940</td>
</tr>
<tr>
<td>2</td>
<td>646</td>
<td>43,700</td>
<td>2.5</td>
<td>2.5</td>
<td>2,750</td>
</tr>
<tr>
<td></td>
<td><strong>Hepatoma 3924A-bearing animal</strong>—tumor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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tracing is, in fact, methyl chenodeoxycholate. Table 4 confirms this by demonstrating retention of radioactivity in repeated recrystallizations of 2 bile acid methyl esters.

**DISCUSSION**

The effects of dietary cholesterol on the regulation of cholesterol synthesis have been studied intensively since Schoenheimer and Breusch first observed a relationship between the two (10, 14). The negative feedback effect, recently reviewed extensively by Dietschy and Wilson (5), is achievable only through cholesterol carried to the liver via the lymph in chylomicron form (15). Moreover, there is evidence that the effect may also be related to tissue concentrations of ester cholesterol, as shown in Table 3 and as suggested earlier (4).

These latter studies, and others (16), suggest that this feedback mechanism, together with the ability of the liver to enhance bile acid synthesis, may in a sense be protective in that it acts to prevent cholesterol accumulation and to promote maximum removal from the organism. The liver, under the impact of a high-cholesterol diet, at once greatly expands its ability to convert hepatic cholesterol to bile acid, while at the same time almost totally ceasing de novo synthesis of sterols. The present study underscores this point by showing that hepatic bile acid formation is intimately tied to the cholesterol feedback mechanism, through which a dual, reciprocating precursor supply for bile acid is made available. There is no quantitatively significant mechanism by which bile acid synthesis is able to proceed from acetate when the synthesis of cholesterol is suppressed, although, as indicated in Table 1, such separate synthesis may occur in minor amounts. The observation that mevalonate, when substituted for acetate, can be converted to bile acid in the liver of the high-cholesterol-fed animals further emphasizes this close relationship, since 3-hydroxy-3-methylglutaryl coenzyme A reductase, the critical dietary cholesterol-sensitive enzyme (13), is then bypassed.

Hepatomas have proved of considerable utility in studies of sterol synthesis in malignant disease, since some varieties of these tumors, particularly slow-growing, well-differentiated ones, are very active in this biochemical function (3). We chose Morris hepatoma 3924A for the present experiments since it is likewise active in sterol synthesis (2), although it is a relatively undifferentiated lesion. It has been noted in earlier work that this hepatoma was not active in sterologenesis (7), but in the past 3 to 4 years we and others have noted an inexplicable enhancement in this function (Ref. 1; J. D. McGarry, personal communication).

Sterol synthesis in virtually all known hepatomas, regardless of rate or degree of tissue differentiation, is, in contrast to liver, unaffected by dietary cholesterol (12). More recently, still another apparently hepatoma-specific regulatory defect in lipogenesis, involving cyclic nucleotides rather than dietary cholesterol, has been described (2).

The present data represent the 1st demonstration of which we are aware that a hepatoma can synthesize bile acid. Further, this synthesis closely reflects the deletion in the dietary cholesterol feedback mechanism characteristic of hepatomas. As shown in Table 3, the hepatoma stores little dietary cholesterol, and its biochemical behavior is in no way demonstrably affected by the presence of dietary cholesterol. Unlike liver, the neoplastic tissue cannot use the dietary substrate in the synthesis of bile acid, of which it nonetheless appears to make a full variety. Beyond demonstrating that the tumor synthesizes cholic and chenodeoxycholic acids, however, no attempt was made to identify precisely which other of these acidic sterols are produced or in what absolute quantities. However, the rates of incorporation of acetate-14C into bile acids by hepatoma 3924A are similar to those of normal liver from a low-cholesterol-fed animal.

Cholesterol synthesized by hepatomas is known to be released into the circulating blood (3). The metabolic fate of bile acids made by these hepatomas forms the basis of a forthcoming report.

**REFERENCES**


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**Table 4**

Confirmation of identity of biosynthetic bile acids by reverse isotope dilution

Recrystallization of bile acid methyl esters isolated from Celite column. The solvent in all cases was methanol, with water added to induce crystal formation.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Source</th>
<th>Wt of crystals (mg)</th>
<th>Radioactivity (dpm)</th>
<th>Specific radioactivity (dpm/100 mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl cholate</td>
<td>Aliquot of methyl cholate fraction</td>
<td>1</td>
<td>25.0</td>
<td>230</td>
</tr>
<tr>
<td></td>
<td>from Celite column</td>
<td>2</td>
<td>23.1</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>14.8</td>
<td>170</td>
</tr>
<tr>
<td>Methyl chenodeoxycholate</td>
<td>Aliquot of methyl chenodeoxycholate fraction</td>
<td>1</td>
<td>44.3</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>from Celite column</td>
<td>2</td>
<td>36.7</td>
<td>270</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>28.0</td>
<td>240</td>
</tr>
</tbody>
</table>
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