Bile Acid Synthesis in Cell Culture*

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Confluent cultures of Hep G2 cells were found to synthesize chenodeoxycholic and chole acids continually. Chenodeoxycholic acid was synthesized at the rate of 58 ± 8.6 μg/96 h, a rate more than 7-fold greater than that for cholic acid. Addition of 5β-cholestan-3α,7α,12α-triol but not the -3α,7α-diol was followed by an increase in cholic acid synthesis, thus indicating a relatively low 12α-hydroxylase activity. Endogenous synthesis of monohydroxy bile acid ester sulfates was found, with maximum rates of 135 and 74 μg/96 h for lithocholic and 3α-hydroxy-5β-choleenoic acids, respectively.

Incubation of Hep G2 cells in medium containing 25% D2O permitted a comparison of the precursor/product relationship of cholesterol with 3β-hydroxy-5β-chole-noic acid. The pattern of incorporation of deuterium was in accordance with that expected, thus allowing the conclusion that this monohydroxy bile acid is derived from cholesterol and should be considered together with chenodeoxycholic and cholic acids as a primary bile acid.

A human liver cell line in culture which continually produces bile acids permits more precise interpretation of data than in vivo studies. In the latter, administered precursors circulate through all the organs, and the excretion of products in bile and/or urine does not necessarily establish the site of their origin. For this reason, uncertainty exists regarding the origin of 3β-hydroxy-5β-cholenoic acid and lithocholic acid and the formation of bile acid glucuronides and ester sulfates. Although isolated liver perfusion and studies of subcellular fractions of liver can reduce the number of variables, they are of relatively short duration and usually focus on specific aspects of the metabolic pathway rather than the interrelationships of cholesterol and bile acid synthesis and the possible role of various lipoprotein fractions that may modulate these activities. The introduction of the Hep G2 cell lines by Knowles and colleagues with the demonstration of many liver-specific metabolic functions (1) including apoprotein synthesis and functioning low density lipoprotein receptors (2, 3) led us to a systematic study of cholesterol and bile acid synthesis including the use of D2O (4) to distinguish the cholesterol formed during the period of study from that present in the cells initially and to delineate its metabolism to bile acids. Although the predominant bile acid was found to be chenodeoxycholic acid, as reported previously by Everson and Polokoff (5), the Hep G2 cell line that we studied had no detectable defects in the side chain oxidation of C27 sterols, and therefore a more normal bile acid profile was obtained.

EXPERIMENTAL PROCEDURES

Lithocholic, deoxycholic, chenodeoxycholic, and cholic acids and their glycine and taurine conjugates were purchased from Supelco, Inc., Bellefonte, PA and 3β-hydroxy-5β-cholenoic acid from Calbiochem. L-[2,3-3H]Lithocholic acid was purchased from Du Pont-New England Nuclear, and 3β-hydroxy-5β-cholestanolic acid was prepared as described previously (6). Both 5β-cholestan-3α,7α-diol and the 12α-triol were synthesized by methods described previously (7). Trihydroxypregnacic acid (3α,7α,12α-trihydroxy-5β-cholestanolic acid) was isolated from the bile of Bombina orientalis (8).

For gas-liquid chromatography-mass spectrometry analysis, the methyl esters were prepared (9) followed by preparation of both the trimethylsilyl ether and acetate derivatives. The reagent MSTFA (N-methyl-N-(trimethylsilyl)trifluoroacetamide, Pierce Chemical Co.) mixed with pyridine was used for preparation of the trimethylsilyl ethers and acetate for the acetates. Using cholic acid methyl ester as a standard, complete derivatization was found to occur when samples were maintained at 120 °C for at least 30 min. The derivatives were injected in the splitless mode onto a 25-m Hewlett-Packard fused silica capillary column coated and then cross-linked with 50% phenylmethyl silicone, film thickness, 0.17 μm; column internal diameter, 0.2 mm. The carrier gas was helium at a head pressure of 27.5 kA. The mass detector was a Hewlett-Packard model 5970 connected to a Hewlett-Packard model 5890 gas-liquid chromatograph. For trimethylsilyl ethers, temperature programming was 0.3 °C/min beginning at 235 °C; and for acetates, programming was at 1 °C/min beginning at 260 °C. The mass detector was used in both the scan (m/z 128-650) and the simultaneous ion-monitoring mode. In the latter mode, the dwell time for each ion was the same.

Hep G2 cells were maintained in minimal essential medium supplemented with penicillin-streptomycin, a standard mixture of amino acids (320-1140, GIBCO), L-glutamine, and 10% heat-inactivated fetal bovine serum. For studies of bile acid synthesis, confluent or almost confluent (85% or more cell growth completed) cell cultures in T-75 flasks (Nunc Laboratory disposable products, Haledon, NJ) were used. The maintenance medium was replaced with 10 ml of the same medium without fetal bovine serum, and the cells were incubated for 96 h. In some studies, the medium was replaced using 99.8% D2O (Merck Isotopes, St. Louis, MO) so that the final D2O/H2O ratio was 0.25. In these studies, the monolayer was washed once with 10 ml of phosphate-buffered saline made up in 25% D2O before medium with the same proportion of D2O was added. In studies of ester sulfation of bile acids, supplementation of the medium with inorganic sulfate was not found to be necessary.

Although a few dead cells were noted to be floating in the medium at 96 h, relatively large numbers did not occur until after this period of time. Therefore, medium was harvested at 96 h by passage through a reverse-phase C18 cartridge (Waters Associates, Milford, MA) followed by elution with methanol. Although linearity of chenodeoxycholic acid production was not determined (5), in some studies medium containing D2O was changed at 24-h intervals, and a progressive increase in the proportion of deuterated cholesterol and bile acids was noted (data not shown). Using radioactive 3β-hydroxy-5β-cholenoic acid and lithocholic acid sulfates added to the medium, virtually complete cartridge extraction and elution were found. In

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some studies, an internal standard consisting of the sodium salt of 3α,7α,12-oxo-5β-cholanoic acid (10 μg) was added to the medium prior to cartridge extraction.

The eluate was utilized for quantitative determination of total bile acids after solvolysis using 2,2-dimethoxypropane and alkaline hydrolysis (9–11), procedures shown previously to give quantitative recovery. In addition, direct determination of bile acid sulfates was done using a method initially described for quantitation of steroid sulfates (12, 13). The reagent consist of methylene blue chloride (Eastman), 250 mg, dissolved in water to which is added 50 g of Na2SO4 and 10 ml of H2SO4, followed by dilution to 1 liter with water. An aliquot of the eluate (0.2 ml in methanol) is added to 1.0 ml of CH3OH followed by the addition of 0.2 ml of methylene blue reagent followed by 1.0 ml of chloroform. The contents are then mixed vigorously and the layers allowed to separate. The upper aqueous layer is aspirated, and the chloroform layer is placed in a 1.0-ml cuvette with a 1-cm light path, and the optical density is determined at 660 nm. After it was established that the silica gel did not contain chromogenic substances, the blank was prepared using 0.2 ml of methanol.

Preliminary studies using standards of the ester sulfates of lithocholic, glycolithocholic, tauroolithocholic, 3β-hydroxy-5-cholenoic, and chenodeoxycholic acids (mono-3-ester) prepared in our laboratory using rat bile chenodeoxycholic acid and chemically the sulfochloric acid indicate that the optical density of the solution was proportional to the sulfate concentration over a final concentration of 1–5 μg/ml. On a molar basis, tauroolithosulfate yielded twice as much color as the ester sulfate or the taurine conjugate of lithocholic acid. Unconjugated and glycine-conjugated bile acids and a glucuronide conjugate of lithocholic acid prepared for use in the laboratory of Professor Koji Nakaniishi, Columbia University, did not yield any color. Also, chemical solvolysis of eluates that yielded chromogenic ethylene blue complexes extractable into chloroform caused a complete loss of color. Thus, we believe that the procedure described for quantitation of steroid sulfates can be used for the direct determination of bile acid sulfates rather than their estimation by difference before and after a solvolysis procedure. Since the method does not distinguish between taurine conjugates and ester sulfates, a chromatographic procedure to separate the different bile acids and their taurine, glycine, and sulfite derivatives is usually necessary.

Thin-layer chromatography was done using Silica Gel G plates (250 μm). To achieve complete resolution of sulfated, conjugated, and unconjugated bile acids, the following three solvent systems were used: (a) 1-butanol/acetic acid/water, 10:1:1; (b) chloroform/methanol/acetic acid/water, 65:24:9:5; (c) isooctane/ethyl acetate/acetic acid, 2:1:1.

At the end of each study, the cells were harvested, and sterols were recovered from the CHCl3 fraction using Folch procedures (14). Following saponification, the enrichment of the cholesterol with deuterium was studied by gas-liquid chromatography-mass spectrometry analysis.

RESULTS

Fig. 1A illustrates the major bile acids recovered from the medium after 96 h of incubation (upper panel) compared with authentic bile acid standards (Fig. 1C). The major peak corresponds to chenodeoxycholic acid, and no bile acids with retention times longer than cholic acid were detected. Attempts to detect C27 bile acids by specific ion monitoring for m/z 410 and 412 were also unsuccessful.

Table I gives the amounts of 3β-hydroxy-5-cholenoic, chenodeoxycholic, and cholic acids which were found after 96 h of incubation. Occasionally, traces of lithocholic acid which were always lower than the amount of 3β-hydroxy-5-cholenoic acid were detected. No monohydroxy bile acids were detected without a solvolysis procedure.

Although addition of 5β-cholestane-3α,7α,12α-triol always significantly increased the proportion of cholic acid (Fig. 1B), total bile acid synthesis did not always increase. Similarly, when 5β-cholestane-3α,7α-diol was added, there was no significant increase in chenodeoxycholic acid production. Nevertheless, in studies utilizing D2O to label the cholesterol and bile acids, analysis of the chenodeoxycholic acid peak revealed much less deuterium incorporation, indicating that the added 5β-cholestane-diol had been metabolized to chenodeoxycholic acid (data not shown).

Fig. 2B indicates the mass spectrum of the peak at 52.05 min compared with that of an authentic standard of 3β-hydroxy-5-cholenoic acid (upper panel).

Fig. 3 illustrates the mass spectrum of the peak obtained between 50 and 52 min after incubation of the Hep G2 cells in medium containing 25% D2O. In addition to the spectrum of 3β-hydroxy-5-cholenoate (Fig. 2A), small peaks associated with major fragment ions such as m/z 370 are noted, confirming the uptake of deuterium in the biosynthesis of this acid. Fig. 4B confirms the deuterium enrichment of this acid after its purification by thin-layer chromatography and preparation of the trimethylsilyl ether.

Fig. 4A illustrates the mass spectrum of the cholesterol isolated from the cells at the end of the study analyzed as the trimethylsilyl ether. In addition to major fragments at m/z 368, small peaks associated with the major fragment indicate the incorporation of deuterium.

The capacity of the Hep G2 cells to sulfate lithocholic acid and 3β-hydroxy-5-cholenoic acid are shown in Table II. More than 100 μg of lithocholic acid was sulfated within 96 h, a quantity greater than that found for 3β-hydroxy-5-cholenoic acid. Addition of 3β-hydroxy-5-cholenoic acid to media containing lithocholic acid caused a reduction in the quantities of both the sulfate derivatives (data not shown). However,
addition of chenodeoxycholic acid did not affect sulfation of the lithocholic acid (Fig. 5), and no sulfated chenodeoxycholic acid was detected (Table II).

**DISCUSSION**

These studies confirm and expand our knowledge concerning bile acid synthesis by the human hepatoma cell line developed by Knowles et al. (1). In agreement with a previous study (5), chenodeoxycholic acid was found to be the predominant bile acid in the media. However, the cell line we obtained apparently had not developed defects in peroxisomal function, and therefore no C27 bile acids were detected.

Further evidence for an absence of defects of side chain oxidation in this cell line was obtained by the addition of 5β-cholenoic acid, which can be attributed to the rapid esterification of chenodeoxycholic acid in the medium, the predominant intermediate in the normal pathway of cheno-

detected lithocholic acid in the medium, the predominant monohydroxy bile acid was the ester sulfate of 3β-hydroxy-5-cholenoic acid, which can be attributed to the rapid esterification that probably occurs following its formation, thus limiting its further metabolism.

It is of interest that esterification of chenodeoxycholic acid to the 3-sulfate did not occur, since this sulfate ester is the major component of urine in cholestatic syndromes (16). The observation implies that a different enzyme catalyzes its

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**TABLE I**

<table>
<thead>
<tr>
<th>Flask no.</th>
<th>3β-OH-5β-cholenoic</th>
<th>Chenodeoxycholic</th>
<th>Cholic</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>511</td>
<td>9.5</td>
<td>69</td>
<td>4.3</td>
<td>74</td>
</tr>
<tr>
<td>609</td>
<td>6.4</td>
<td>27</td>
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</tr>
<tr>
<td>612</td>
<td>6.9</td>
<td>26</td>
<td>5.5</td>
<td>39</td>
</tr>
<tr>
<td>561</td>
<td>5.4</td>
<td>48</td>
<td>9.7</td>
<td>63</td>
</tr>
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<tr>
<td>560</td>
<td>8.9</td>
<td>62</td>
<td>10</td>
<td>81</td>
</tr>
<tr>
<td>557</td>
<td>1.6</td>
<td>78</td>
<td>9.2</td>
<td>89</td>
</tr>
<tr>
<td>555</td>
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<td>93</td>
<td>11</td>
<td>106</td>
</tr>
<tr>
<td>517</td>
<td>6.4</td>
<td>77</td>
<td>14</td>
<td>97</td>
</tr>
</tbody>
</table>

Mean ± S.E. 5.4 ± 1.0 58 ± 7.6 8.7 ± 1.0 72 ± 8.0

5β-Cholenoic acid (7aα,12α-triol added) μg/96 h/dish

<table>
<thead>
<tr>
<th>Flask no.</th>
<th>5β-Cholenoic acid (7αα,12α-diol added) μg/96 h/dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>521</td>
<td>3.4 69 13 85</td>
</tr>
<tr>
<td>646</td>
<td>4.5 73 11 89</td>
</tr>
<tr>
<td>647</td>
<td>2.1 58 9.7 70</td>
</tr>
</tbody>
</table>

Mean ± S.E. 3.3 ± 0.7 67 ± 4.5 11.4 ± 1.1 81 ± 5.8

* Each dish contained 3–4 × 10⁵ cells.

<sup>a</sup> 50 μg dissolved in 20 μl of dimethylformamide was added to the media at zero time.

<sup>b</sup> p = 0.107.

<sup>c</sup> p < 0.001.

<sup>d</sup> p = 0.36.

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**FIG. 2.** Mass spectrum of peak 2 compared with the methyl 3β-acetoxy-5-cholenoate. Comparison of the mass spectrum of an authentic standard (panel A) with the bile acid isolated from the media (panel B) indicates no major differences. Panel C illustrates the total ion current obtained from peaks 2, 4, and 5 (A) compared with the internal standard (peak 6, 101 min).

**FIG. 3.** Mass spectrum of methyl 3β-hydroxy-5-cholenoate from Hep G2 cells grown in medium containing 25% D₂O. As shown in the bottom panel, the peak (total ion current) is asymmetrical because of the earlier retention times of the deuterated species (upper panel). Small peaks following m/z 370 confirm the uptake of deuterium during biosynthesis of the bile acid.
indicates that the predominant enriched species, hydroxy-5-cholenoic acid with that of deuterated cholesterol esterification and that it is not expressed in this cell line or, alternatively, that esterification of this bile acid normally occurs in the kidney or other organ.

Comparison of the mass spectral pattern of deuterated 3β-hydroxy-5-cholenoic acid with that of deuterated cholesterol indicates that the predominant enriched species, m/z 375 and 376, are 5 and 6 mass units greater than the major ion fragment at m/z 370, in contrast to m/z 376 and 377, which are 8 and 9 mass units greater, respectively, that the major ion fragment at m/z 368. The findings are in accordance with the assignment of deuterium atoms derived from water to cholesterol that it originates in the liver together with chenodeoxycholic and cholic acids.

A human liver cell line that continually synthesizes the naturally occurring bile acids provides unique opportunities for study. In contrast to subfractions of liver cell homogenates which permit the study of individual steps in bile acid synthesis, it is possible to evaluate the entire metabolic sequence of events, the interrelationships of cholesterol and bile acid intermediates and xenobiotics.

**TABLE II**

**Sulfation of lithocholic and 3β-hydroxy-5-cholenoic acids by Hep G2 cells**

<table>
<thead>
<tr>
<th>Bile acid</th>
<th>Sulfation*</th>
<th>µg sulfate/µCi</th>
<th>µg sulfate/96 h/dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lithocholic</td>
<td>5</td>
<td>5.3</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>105</td>
<td>54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>400 (3)</td>
<td>135 ± 17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3β-Hydroxy-5-cholenoic</td>
<td>6.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>104</td>
<td>58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>400 (2)</td>
<td>93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chenodeoxycholic</td>
<td>74 ± 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>None detected</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Stock solution prepared in dimethyl formamide. Volume of solvent added ranged from 10 to 30 µl.
* Each dish contained 3–4 × 10⁶ cells.
* Number of studies is indicated in parentheses.

FIG. 4. Comparison of the mass spectrum of the trimethylsilyl ethers of 1-deuterated cholesterol to deuterated methyl 3β-hydroxy-5-cholenoate. Compared with the major deuterium-enriched cholesterol peak (m/z 376) which is 8 mass units greater than the corresponding protium peak (m/z 375), the bile acid has a major enriched peak at m/z 375 which is only 5 mass units greater than the corresponding protium peak (m/z 370) and indicates a loss of three deuteriums during oxidation of the side chain from C27 to C24.

**FIG. 5.** Sulfation of lithocholic acid by Hep G2 cells. Both the optical density and radioactivity of the methylene blue complexes in chloroform were determined. The absence of a change in either the rate of sulfation or the ratio of µg of sulfate/µCi indicates that sulfation of chenodeoxycholic acid did not occur.

REFERENCES