Pathogenesis of gallstones in Crohn's disease
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Citation for published version (APA):

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Chapter 4

URSODEOXYCHOLIC ACID AND CHOLESTEROL INDUCE ENTEROHEPATIC CYCLING OF BILIRUBIN IN RODENTS
Background & Aims: Oral ursodeoxycholic acid (UDCA) and cholesterol cause bile salt malabsorption; the former by competition for, and the latter by down-regulation of ileal bile acid transporters. Because ileectomy in rats induces enterohepatic cycling of bilirubin, we tested the hypothesis that dietary steroids might have the same effect.

Methods: We fed male inbred C57L/J mice and Sprague-Dawley rats low doses of UDCA, chenodeoxycholic acid (CDCA) or cholesterol added to laboratory chow with simultaneous chow-fed controls. After 1 week (mice) or 2 weeks (rats), we measured indices of bile salt malabsorption and enterohepatic cycling of bilirubin, including bilirubin secretion rates into bile, serum and intestinal bilirubin and bile salt levels as well as urobilinogen in cecum, large intestine and feces.

Results: Dietary UDCA and cholesterol, but not CDCA, significantly increased bilirubin secretion rates into bile. In UDCA-fed mice, gallbladder biles contained increased levels of bilirubin conjugates and unconjugated bilirubin, and in 60%, granules of amorphous calcium bilirubinate precipitated. Dietary cholesterol and bile acids, particularly UDCA, increased cecal bile salts, unconjugated bilirubin and urobilinogen concentrations, and decreased fecal bilirubin outputs, consistent with colonic absorption.

Conclusions: By causing bile salt malabsorption, dietary UDCA and cholesterol induce enterohepatic cycling of bilirubin.
Introduction

In the mouse, bile acids, usually cholic acid and cholesterol, are required for dietary induction of cholesterol gallstones.\textsuperscript{1,2} Surprisingly, several animal models of pigment gallstones also require diets high in cholesterol,\textsuperscript{3-6} but a rational pathophysiological explanation is lacking. Further, it is known that cholesterol gallstones can acquire calcified shells during ursodeoxycholic acid (UDCA) therapy,\textsuperscript{7-12} and less frequently with chenodeoxycholic acid (CDCA) therapy\textsuperscript{11,12} and surface deposits of amorphous black pigment precipitates have been associated with gallstones resistant to dissolution therapy\textsuperscript{13,14}.

In recent work, we suggested that bilirubin cycles enterohepatically after ileal resection in the rat,\textsuperscript{15} providing a possible pathophysiological mechanism for pigment gallstone formation in patients with ileal disease, bypass or resection. We proposed that enterohepatic cycling of bilirubin occurs because of bile salt malabsorption, which elevates colonic bile salt levels, solubilizes unconjugated bilirubin (UCB) and promotes its passive absorption from the intestine.\textsuperscript{15} UDCA, in the ionized form, as well as its taurine (T) and glycine (G) conjugates, can compete with endogenous bile salts for ileal bile acid transporters (IBAT), leading to bile salt malabsorption.\textsuperscript{16-18} Furthermore, dietary cholesterol may have a “cholestyramine-like” effect in both rats\textsuperscript{19} and humans,\textsuperscript{20} leading to bile salt malabsorption and increased colonic bile salt concentrations, explained now by down-regulation of IBAT gene expression at the transcriptional level.\textsuperscript{21}

In this work, we tested the hypothesis that administration of either oral UDCA or cholesterol induces enterohepatic cycling of endogenous bilirubin. We added UDCA, chenodeoxycholic acid (CDCA), or cholesterol to the chow diet of mice and rats and after 1 or 2 weeks, respectively, we found that total bilirubin secretion rates into bile increased, but chow- or CDCA-fed animals did not exhibit this effect. UDCA and cholesterol increased cecal bile salt, UCB and urobilinogen levels significantly and these changes correlated positively with increased plasma UCB and urobilinogen concentrations and decreased fecal bilirubin excretion rates. These results suggest that UDCA and cholesterol, by causing bile salt malabsorption, induce enterohepatic cycling of bilirubin.
Material and Methods

Chemical reagents

Ultrapure samples of UDCA and CDCA from Diamalt Aktiengesellschaft (Munich, Germany) were donated generously by Dr. Herbert Falk (Freiburg, Germany). Authentic samples of the T- and G-conjugates of α-, β-, and ω-muricholic acid, used as high performance liquid chromatography (HPLC) standards, were obtained from Tokyo Tanabe, Ltd., Tokyo, Japan (courtesy of Mr. H. Sugata). Purified reagent grade cholesterol was obtained from Avanti Polar Lipids (Alabaster, AL).

Animals

Male Sprague-Dawley rats (Charles River Breeding Laboratories Inc., Wilmington, MA) of 351 ± 10 g (mean ± S.E.) body weight (B.W.) and male C57L/J inbred mice (The Jackson Laboratory, Bar Harbor, ME) of 27.9 ± 0.3 g B.W., were studied. Animals were housed in screen-floor cages at 23°C, and daylight cycles were 12 hours in duration. The rodents were fed a pulverized laboratory chow diet (Rodent-lab Diet # 5001, Purina Mills, Inc., St. Louis, MO) for at least one week prior to administering the experimental diet. All aspects of the study conformed to accepted criteria for the care and use of laboratory animals, and experimental protocols were approved by the Harvard Medical Area Standing Committee on Animals. Euthanasia was consistent with recommendations of the American Veterinary Medical Association.

Experimental Design

In preliminary studies, we found that C57L/J mice, which are a genetic model for cholesterol gallstone formation, did not tolerate 1% (wt/wt %) dietary bile, in that B.W. declined approximately 10% within a week, resulting in a high mortality rate. Lower doses were well tolerated, so we fed C57L/J mice laboratory chow containing 0.1, 0.25 and 0.5% of UDCA or CDCA for one week, while control animals were fed chow without bile acids. In another set of experiments, we fed mice chow diets containing 0.5, 1, 2 and 3% cholesterol prepared by mixing cholesterol solubilized in hot ethanol with pulverized chow and subsequently evaporating the solvent at 80°C. The chow diet alone, which contained trace
(0.02% by wt) cholesterol, was fed to control animals. After 1 week of feeding, short-term biliary washout studies were performed.\textsuperscript{15}

To quantify whether bile salt malabsorption was induced by UDCA, CDCA or cholesterol, the cecum and 5 cm of proximal large intestine were resected in mice and their contents pooled.\textsuperscript{15} Cecal and proximal large bowel contents were aspirated, frozen rapidly, freeze-dried, and stored at -70°C for bile salt analyses.

In another set of experiments, we determined plasma UCB and urobilinogen concentrations, as well as 24-hour bile salt, bilirubin and urobilinogen excretion rates in feces. In parallel experiments, we fed C57L/J mice 0.25% bile acids, 1% cholesterol or laboratory chow for 1 week, and small intestine, cecum and large intestine were resected for measurement of bile salt, bilirubin and urobilinogen levels. Blood was obtained by cardiac puncture for determination of plasma UCB, urobilinogen, hemoglobin, hematocrit and indices of cholestasis and liver injury.

We fed Sprague-Dawley rats a pulverized laboratory chow diet containing 0.04, 0.08, 1 and 2% (wt %) of UDCA or CDCA as well as 0.5% or 1% cholesterol, with laboratory chow-fed controls. At 2 weeks, measurement of biliary bilirubin and lipid secretory rates were also performed by acute "washout" for 30 min after bile duct cannulation. Serum UCB concentrations and 24-hour fecal UCB excretion rates were determined.

**Bile Duct Cannulation and “Washout” of Bile**

We anesthetized both mice and rats with intraperitoneal pentobarbital (35 mg/kg B.W.) and performed a midline abdominal incision. Each animal’s body temperature was maintained and monitored at 37°C using a heating lamp. We constructed an acute biliary fistula by cannulating the bile duct of rats with a PE-10 polyethylene catheter (ID of 0.28 mm, an OD of 0.61 mm), the size of which minimized dead space and prevented acute cholestasis.\textsuperscript{15} The biliary catheter was sutured to the abdominal wall, and after closure of the abdomen, the rats were placed in a restraining cage. In the case of mice, gallbladder biles were aspirated completely and examined by light-microscopy for calcium bilirubinate or other precipitates. After ligation of the distal common bile duct, a catheter was inserted through the fundus of the gallbladder and wedged into the cystic duct. The abdomen was then covered with aluminum foil. Employing a fraction collector, two 15 min aliquots of hepatic bile were collected in
tared tubes over ice. Tubing and glassware were covered with aluminum foil, and lights were dimmed to protect bilirubin from photodegradation. Bile volumes were determined gravimetrically, assuming a specific gravity of unity. Fresh bile was analyzed within 2 hrs for bile pigments by HPLC (see below) and then frozen under argon and stored at -20°C for later analysis of biliary lipids. Biliary lipid and bilirubin secretion rates were calculated multiplying biliary concentrations by bile volumes. We verified that two isomers of bilirubin monoglucuronide (BMG), were the principal bile pigments in the mouse\textsuperscript{23}, and bilirubin diglucuronide (BDG), the principal bile pigment in the rat,\textsuperscript{23} and their molar ratios were calculated. At the end of each experimental period, animals were euthanized with an overdose of diethyl ether or pentobarbital.

**Analytical Methods**

Biliary bilirubins were separated and quantified by HPLC.\textsuperscript{24} For determinations of bilirubins in serum, intestine and feces, ion-pair extraction was carried out with 0.1 mol/L of methanolic di-n-octylamine acetate,\textsuperscript{25} and after alkaline methanolysis and extraction into chloroform, UCB was measured by HPLC.\textsuperscript{26} Urobilinogens were quantified by direct spectrophotometry of a zinc complex.\textsuperscript{27} Total bile salts were assayed by the 3α-hydroxysteroid dehydrogenase method.\textsuperscript{28} Biliary phospholipids were measured with the Bartlett assay\textsuperscript{29} and biliary cholesterol was assayed by HPLC.\textsuperscript{30} Individual bile salts in bile were separated and quantified according to the HPLC procedure of Rossi et al.\textsuperscript{31} We did not attempt to fractionate muricholates or ursodeoxycholates in rat bile into their Δ\textsuperscript{22} homologs.\textsuperscript{32} Cecal and colonic bile salts were extracted with t-butanol/water (50:50, v/v) using 40 to 70 mg portions of dry contents\textsuperscript{33} and assayed enzymatically.\textsuperscript{28}

Because of the potential toxicity of bile acid feeding in rodents,\textsuperscript{16} weight of food intake and B.W. were recorded every other day before and during the experimental period. Since a cholesterol-enriched diet may increase liver weight due to accumulation of cholesteryl esters and triglycerides,\textsuperscript{22,34} we resected and weighed the livers of animals following sacrifice.
Statistical Analyses

All values are expressed as means ± SEM. For multiple comparisons of data from different animal groups, we used a one-way nonparametric test (analysis of variance) and P values less than 0.05 were judged to be significant.

Results

MICE: BILE ACID AND CHOLESTEROL FEEDING EXPERIMENTS

Condition of Animals

None of the experimental diets influenced food intake nor mean rodent B.W. appreciably (data not shown). All animals remained healthy throughout the experimental period and none developed diarrhea. Mean liver weights were comparable to those of chow-fed controls and were unaffected by either bile acid or cholesterol feeding (data not shown).

Serum indices of hemolysis, liver toxicity and cholestasis

Control hemoglobin and hematocrit levels of pooled blood samples (n = 9) were 14.6 g/dl and 47.9% respectively. The values were similar (14.8-15.3 g/dl) after one week of feeding 0.25% (wt/wt) bile acids or 1% (wt/wt) cholesterol diets, thereby excluding significant hemolysis. Compared to control levels, administration of UDCA and CDCA resulted in small increases in alanine aminotransferase (13 ± 0.5 to 26 ± 6 U/L) and alkaline phosphatase (25 ± 5 to 41 ± 1 U/L) but the differences in these indices of liver toxicity and cholestasis, respectively, were not statistically significant.

Individual bile salt species in hepatic bile

Table 1 lists percentages of individual bile salt species in hepatic biles of mice at 1 week of bile acid, cholesterol or laboratory chow feeding (control). In chow-fed mice, bile contained predominantly taurocholate (55.2 ± 1.0%) and tauro β-muricholate (Tβ-MC) (34.5 ± 1.7%), with only small amounts (2.9 ± 1.7%) of tauro α-muricholate (Tα-MC) and other bile salts present. In contrast, the bile salt species of mice on 0.1% and 0.5% UDCA were markedly enriched in TUDC (66.3 ± 2.2 and 87.8 ± 2.0%, respectively). The bile salt pool of mice on
the lowest CDCA dose (0.1%) consisted predominantly of Tβ-MC and Tα-MC (approximately 65% of total) and only 9% taurochenodoxycholate (TCDC), indicating efficient biotransformation of low dose CDCA into muricholates. In contrast, the bile salt pool of mice ingesting the higher CDCA dose was enriched (approximately 80% of total) with TCDC, Tα-MC and taurodeoxycholate (TDC). The bile salt pattern of mice fed chow and cholesterol-containing diets remained relatively stable with no significant changes in Tβ-MC and Tα-MC as well as taurocholate levels (Table 1).

### Table 1. C57L/J Mice: Individual Bile Salt Species in Hepatic Bile as Percentages of Total Bile Salt Composition After 1 Week on Diet

<table>
<thead>
<tr>
<th>Diet*</th>
<th>n</th>
<th>Tβ-MC</th>
<th>Tα-MC</th>
<th>TCDC</th>
<th>GUDC</th>
<th>TC</th>
<th>TDC</th>
<th>TCDC</th>
<th>GCDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>34.5 ± 1.7</td>
<td>2.9 ± 1.7</td>
<td>26.8 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>55.2 ± 1.0</td>
<td>2.7 ± 0.4</td>
<td>1.0 ± 0.1</td>
<td>—</td>
</tr>
<tr>
<td>UDCA</td>
<td>6</td>
<td>12.5 ± 1.3</td>
<td>6.3 ± 0.2</td>
<td>66.3 ± 2.2</td>
<td>0.7 ± 0.1</td>
<td>9.2 ± 1.4</td>
<td>1.9 ± 0.4</td>
<td>2.8 ± 0.2</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>0.5</td>
<td>3</td>
<td>4.1 ± 0.4</td>
<td>2.0 ± 0.5</td>
<td>87.8 ± 2.0</td>
<td>1.2 ± 0.6</td>
<td>1.3 ± 0.4</td>
<td>0.4 ± 0.4</td>
<td>3.3 ± 1.2</td>
<td>—</td>
</tr>
<tr>
<td>CDCA</td>
<td>6</td>
<td>40.6 ± 0.7</td>
<td>24.0 ± 3.9</td>
<td>6.5 ± 0.7</td>
<td>0.5 ± 0.2</td>
<td>18.2 ± 3.0</td>
<td>1.5 ± 0.2</td>
<td>8.7 ± 0.8</td>
<td>—</td>
</tr>
<tr>
<td>0.5</td>
<td>6</td>
<td>4.9 ± 1.0</td>
<td>23.3 ± 3.9</td>
<td>8.1 ± 1.0</td>
<td>—</td>
<td>4.3 ± 3.4</td>
<td>9.8 ± 9.8</td>
<td>48.8 ± 8.8</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1</td>
<td>36.1</td>
<td>9.6</td>
<td>3.7</td>
<td>2.5</td>
<td>42.6</td>
<td>4.2</td>
<td>1.2</td>
<td>—</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>38.9</td>
<td>7.9</td>
<td>2.2</td>
<td>—</td>
<td>47.5</td>
<td>2.3</td>
<td>1.2</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>43.1</td>
<td>11.6</td>
<td>2.4</td>
<td>2.4</td>
<td>36.2</td>
<td>3.1</td>
<td>1.2</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>43.5</td>
<td>4.8</td>
<td>5.3</td>
<td>—</td>
<td>43.0</td>
<td>2.5</td>
<td>1.0</td>
<td>—</td>
</tr>
</tbody>
</table>

**NOTE.** Bile salt species were measured by HPLC (Rossi et al.11). Data given as means ± SEM.

n, no. of animals; GUDC, glyoursodeoxycholate; TC, taurocholate; TDC, taurodeoxycholate; GCDC, glycochenodeoxycholate; -, not found or nonquantifiable trace.

*Dietary additions and weight percentages administered.

**Gallbladder bile; physical state and bile pigment composition**

By polarizing light-microscopy, we observed amorphous calcium bilirubinate precipitates\(^{35}\) in gallbladder biles of 4 of 6 mice on the 0.5% UDCA diet, in 1 of 6 animals on the 0.5% CDCA diet, and in none of chow-fed controls. Over the 1-wk period, no animal (total n = 66) developed pigment (or cholesterol) gallstones. Unfortunately, in cholesterol-fed mice, gallbladder volumes were too small for unambiguous light-microscopic examination. Table 2 summarizes gallbladder bile bilirubin levels in mice at 1 wk of bile acid, cholesterol and laboratory chow feeding. In UDCA-fed mice, total bilirubin levels in gallbladder bile were elevated but fell short (\(P = 0.15\)) of statistical significance, whereas biliary UCB concentrations were increased significantly (\(P < 0.05\)) compared to the other groups. In all groups, BMG/BDG molar ratios were similar with ratios typical for the mouse.\(^{23}\)
Table 2. C57L/J Mice: Total and Unconjugated Bilirubin Levels in Gallbladder Bile After 1 Week on Diet

<table>
<thead>
<tr>
<th>Diet</th>
<th>%</th>
<th>n</th>
<th>Total bilirubin (μmol/L)</th>
<th>UCB (μmol/L)</th>
<th>BMG/BDG ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>9</td>
<td>135 ± 16</td>
<td>2.6 ± 0.2</td>
<td>5.8 ± 0.4</td>
</tr>
<tr>
<td>UDCA</td>
<td>0.25</td>
<td>9</td>
<td>183 ± 16</td>
<td>3.5 ± 0.3b</td>
<td>5.5 ± 0.2</td>
</tr>
<tr>
<td>CDCA</td>
<td>0.25</td>
<td>6</td>
<td>142 ± 19</td>
<td>2.4 ± 0.3</td>
<td>6.3 ± 0.4</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1</td>
<td>9</td>
<td>127 ± 15</td>
<td>2.8 ± 0.1</td>
<td>6.6 ± 0.6</td>
</tr>
</tbody>
</table>

NOTE. Total bilirubin and UCB in bile were measured by HPLC (Spivak and Yuey). Data given as means ± SEM. n, no. of animals.

°Dietary additions and percentages administered.
Statistically significant difference (P < 0.05) vs. control, CDCA, and cholesterol-fed mice.

Biliary lipid and bilirubin secretion rates

Table 3 displays biliary bilirubin and lipid secretion rates of mice during the first (0-15 min) washout period reflecting minimal perturbation of the enterohepatic circulation of bile salts. Bile salt secretion rates as well as phospholipid and cholesterol secretion rates increased significantly (P < 0.05) in mice fed higher UDCA and CDCA doses, compared to controls and lower dose bile acid feeding. Biliary bile salt secretion rates on cholesterol-enriched diets decreased non-significantly compared with controls, and biliary phospholipid secretion rates were either unaffected or increased significantly (Table 3). Compared with chow-fed controls, cholesterol secretion rates increased markedly (P < 0.05) in mice fed the highest UDCA and CDCA doses as well as those groups fed 1-3% cholesterol. Compared with controls, bilirubin secretion rates were significantly higher in mice fed 0.5% UDCA or CDCA or 1% cholesterol. The BMG/BDG ratios were not influenced appreciably by any bile acid or dietary cholesterol regimen and fell in the 8-11 range (data not displayed). The salient features of the secretory data are displayed for purposes of comparison in Figs. 1 and 2.
Table 3. C57L/J Mice: Bilirubin and Biliary Lipid Secretion Rates During Acute Biliary "Washout" Performed After 1 Week on Diet

<table>
<thead>
<tr>
<th>Diet</th>
<th>%</th>
<th>n</th>
<th>Bilirubin</th>
<th>Bile salt</th>
<th>Phospholipid</th>
<th>Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(nmol/h • 100 g body wt)</td>
<td>(μmol/h • 100 g body wt&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>(μmol/h • 100 g body wt&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>(μmol/h • 100 g body wt&lt;sup&gt;−1&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>10</td>
<td>61.9 ± 6.7</td>
<td>18.3 ± 1.4</td>
<td>0.68 ± 0.04</td>
<td>121 ± 5</td>
</tr>
<tr>
<td>UDCA</td>
<td>0.1</td>
<td>5</td>
<td>81.5 ± 10.8</td>
<td>17.1 ± 3.6</td>
<td>0.62 ± 0.07</td>
<td>90 ± 10</td>
</tr>
<tr>
<td>0.25</td>
<td>5</td>
<td></td>
<td>96.9 ± 14.0</td>
<td>41.4 ± 5.5</td>
<td>0.67 ± 0.02</td>
<td>193 ± 29</td>
</tr>
<tr>
<td>0.5</td>
<td>5</td>
<td></td>
<td>174.4 ± 30.2</td>
<td>33.4 ± 5.1</td>
<td>0.99 ± 0.07</td>
<td>299 ± 42</td>
</tr>
<tr>
<td>CDCA</td>
<td>0.1</td>
<td>6</td>
<td>115.5 ± 25.0</td>
<td>18.4 ± 2.0</td>
<td>0.63 ± 0.10</td>
<td>101 ± 13</td>
</tr>
<tr>
<td>0.25</td>
<td>6</td>
<td></td>
<td>66.0 ± 9.5</td>
<td>25.9 ± 3.8</td>
<td>1.15 ± 0.11</td>
<td>200 ± 19</td>
</tr>
<tr>
<td>0.5</td>
<td>6</td>
<td></td>
<td>110.5 ± 10.7</td>
<td>49.3 ± 9.6</td>
<td>1.99 ± 0.43</td>
<td>441 ± 69</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.5</td>
<td>6</td>
<td>62.1 ± 7.5</td>
<td>12.4 ± 3.2</td>
<td>1.64 ± 0.06</td>
<td>149 ± 14</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>6</td>
<td>103.0 ± 18.0</td>
<td>15.9 ± 2.3</td>
<td>0.66 ± 0.12</td>
<td>260 ± 23</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6</td>
<td>73.5 ± 8.5</td>
<td>12.5 ± 0.8</td>
<td>0.86 ± 0.04</td>
<td>242 ± 13</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5</td>
<td>52.3 ± 4.5</td>
<td>15.1 ± 2.2</td>
<td>0.93 ± 0.06</td>
<td>307 ± 18</td>
</tr>
</tbody>
</table>

NOTE. Total bilirubin was measured by HPLC (Spivak and Yuey<sup>24</sup>), total bile salts and phospholipids were measured by enzymatic methods (Turley and Dietschy<sup>28</sup> and Bartlett<sup>29</sup>), and cholesterol was measured by HPLC (Vercaens et al.<sup>30</sup>). Data given as means ± SEM.

n. no. of animals in each group.

<sup>a</sup>Dietary additions and percentages administered.

<sup>b</sup>Timing of biliary washout from insertion of cannula.

<sup>c</sup>Statistically significant differences (P < 0.05) vs. control values.

UDCA or CDCA. Although there is a step-wise increase in bilirubin secretory rates with increments in dietary bile acid levels, the rates are increased significantly (P < 0.05) only with 0.5% UDCA, and to a lesser extent with 0.5% CDCA compared with chow-fed controls or animals fed 0.25% CDCA. On low dose (0.1%) CDCA (Table 1), when the bile salt species consisted predominantly of Tβ-MC and Tα-MC bilirubin secretion rates are appreciably higher, but comparable to identical dose UDCA.

Figure 2 plots mean bilirubin secretion rates as functions of progressive increments in the dietary cholesterol dose in mice. Compared with chow-fed controls, 1% cholesterol increases bilirubin secretion rates significantly (p < 0.05), whereas higher or lower cholesterol doses do not have this effect.

**Plasma and intestinal bile salt, bilirubin and urobilinogen levels**

With 0.5% UDCA or CDCA, colonic bile salt levels per g of dry large intestinal content increased tenfold (33.8 and 27.6 μmoles/g) compared to chow-fed controls (3.0 μmoles/g). In
Bile Acid and Cholesterol Administration in Rodents

animals fed 0.5, 1, 2 and 3% in dietary cholesterol, colonic bile salt concentrations increase incrementally, respectively, to 3.6, 5.3, 4.5 and 6.9, µmoles/g (dry large intestinal content).

Figure 1. Total bilirubin secretion rates (in nanomoles per hour per 100 g body wt) during 0-15 minutes of biliary washout performed in inbred C57L/J mice after 1 week of laboratory chow, UDCA, or CDCA administration. □, Chow-fed animals; ■, UDCA-fed animals; •, CDCA-fed animals. *Statistically significant differences (P < 0.05) between groups (therefore, 0.5% UDCA is significantly different from 0 [chow-fed] and 0.25% [CDCA], and 0.5% CDCA is significantly different from 0 [chow-fed]. Data given as means ± SEM.

Figure 2. Total bilirubin secretion rates (in nanomoles per hour per 100 g body wt) during 0-15 minutes of biliary washout performed after 1 week of feeding cholesterol to inbred C57L/J mice. □, Chow-fed animals. ■, cholesterol-fed animals. *Statistically significant differences (P < 0.05) between groups (therefore, total bilirubin secretion rate into bile is significantly greater with 1% cholesterol compared with chow [0%] and 3% cholesterol). Data given as means ± SEM.

Table 4 lists small intestinal, cecal and large intestinal concentrations of bile salts and bilirubins in mice after 0.25% bile acid, 1% cholesterol or chow diets for 1 wk. Compared with controls, dietary bile acids and cholesterol cause no change in small intestinal levels, but induce a fourfold increase in cecal and an eightfold increase in large intestinal bile salt levels, respectively. Cecal levels of bilirubins were increased significantly in mice fed UDCA and cholesterol but not by CDCA (Table 4). In the large intestine, total bilirubin levels are not significantly different from control values in any steroid-fed group, but as expected, bile salt levels are elevated with both UDCA and CDCA diets. Cecal bilirubin levels are increased significantly in animals fed UDCA and cholesterol, compared with CDCA. In contrast to CDCA and cholesterol diets, large intestinal bilirubin levels decrease markedly with UDCA diets compared with cecal concentrations (Table 4).
Neither UCB nor urobilinogen in small intestinal contents differ between any mouse group. However, UCB levels in the cecum are elevated significantly with all diets compared to controls, paralleling the elevated total cecal bilirubin concentrations (Table 4). Not only was this elevation most marked for mice fed UDCA or cholesterol, but cecal urobilinogen levels in both cases were elevated significantly compared to CDCA, and doubling control values. In the large intestine, UCB levels decreased again, reaching control levels in the case of UDCA (Table 4) and correlating inversely with a significant increase in plasma UCB. In contrast, colonic UCB levels in CDCA- and cholesterol-fed mice remained unchanged compared with the cecum and the plasma UCB concentration showed less marked increases. The levels of urobilinogen, the principal intestinal catabolite of bilirubin, are significantly increased (P < 0.05) in the cecum of UDCA- and 1% cholesterol-fed mice but significantly decreased in the large intestines of UDCA-fed groups. In mice fed 1% cholesterol (Table 4), the plasma urobilinogen concentration doubled that of controls, suggesting marked absorption of urobilinogens from colonic contents.

**Fecal bile salt, bilirubin and urobilinogen excretion**

Table 5 summarizes fecal excretion rates of bile salts, bilirubin (total and UCB), and urobilinogen in mice at 1 wk of feeding. On cholesterol and bile acid diets, the fecal bile salt excretion rates were almost eight- and twentyfold increased, respectively, compared with control animals. In contrast, fecal excretion rates of total bilirubins, and UCB were decreased appreciably on all diets, just missing statistical significance (P < 0.06) in the case of mice fed 0.25% UDCA. Fecal urobilinogen excretion rate was reduced markedly in animals on the 1% cholesterol-containing diet, consistent with the elevation in plasma urobilinogen (Table 4).
Table 4. C57L/J Mice: Intestinal and Plasma Concentrations of Bile Salts, Bilirubin, UCB, and Urobilinogen After 1 Week on Diet

<table>
<thead>
<tr>
<th>Group</th>
<th>Bile salts (µmol/100 g body wt)</th>
<th>Bilirubin (µmol/100 g body wt)</th>
<th>UCB (nmol/100 g body wt)</th>
<th>Urobilinogen (nmol/100 g body wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n = 9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small intestine</td>
<td>11.2 ± 2.5</td>
<td>0.16 ± 0.03</td>
<td>16 ± 4</td>
<td>70 ± 8</td>
</tr>
<tr>
<td>Cecum</td>
<td>0.5 ± 0.8</td>
<td>0.04 ± 0.003</td>
<td>12 ± 2</td>
<td>118 ± 24</td>
</tr>
<tr>
<td>Large intestine</td>
<td>0.6 ± 0.2</td>
<td>0.07 ± 0.01</td>
<td>33 ± 7</td>
<td>129 ± 18</td>
</tr>
<tr>
<td>Plasma</td>
<td>-</td>
<td>-</td>
<td>32 ± 5</td>
<td>673 ± 119</td>
</tr>
<tr>
<td>UDCA 0.25% (n = 9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small intestine</td>
<td>12.2 ± 0.5</td>
<td>0.16 ± 0.03</td>
<td>18 ± 3</td>
<td>83 ± 31</td>
</tr>
<tr>
<td>Cecum</td>
<td>3.3 ± 0.5</td>
<td>0.17 ± 0.02</td>
<td>77 ± 17</td>
<td>244 ± 24</td>
</tr>
<tr>
<td>Large intestine</td>
<td>2.4 ± 0.5</td>
<td>0.07 ± 0.01</td>
<td>34 ± 8</td>
<td>68 ± 13</td>
</tr>
<tr>
<td>Plasma</td>
<td>-</td>
<td>-</td>
<td>120 ± 40</td>
<td>587 ± 27</td>
</tr>
<tr>
<td>CDCA 0.25% (n = 9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small intestine</td>
<td>16.1 ± 2.4</td>
<td>0.20 ± 0.03</td>
<td>20 ± 10</td>
<td>51 ± 9</td>
</tr>
<tr>
<td>Cecum</td>
<td>4.2 ± 1.7</td>
<td>0.10 ± 0.02</td>
<td>56 ± 9</td>
<td>138 ± 23</td>
</tr>
<tr>
<td>Large intestine</td>
<td>2.2 ± 0.1</td>
<td>0.11 ± 0.01</td>
<td>60 ± 11</td>
<td>144 ± 20</td>
</tr>
<tr>
<td>Plasma</td>
<td>-</td>
<td>-</td>
<td>90 ± 20</td>
<td>556 ± 13</td>
</tr>
<tr>
<td>Cholesterol 1% (n = 9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small intestine</td>
<td>15.7 ± 7.3</td>
<td>0.16 ± 0.09</td>
<td>44 ± 11</td>
<td>96 ± 22</td>
</tr>
<tr>
<td>Cecum</td>
<td>1.9 ± 0.3</td>
<td>0.13 ± 0.02</td>
<td>62 ± 13</td>
<td>227 ± 38</td>
</tr>
<tr>
<td>Large intestine</td>
<td>1.2 ± 0.2</td>
<td>0.14 ± 0.04</td>
<td>71 ± 19</td>
<td>149 ± 19</td>
</tr>
<tr>
<td>Plasma</td>
<td>-</td>
<td>-</td>
<td>115 ± 25</td>
<td>1006 ± 26</td>
</tr>
</tbody>
</table>

NOTE. Bile salts were measured by an enzymatic method (Turley and Dietschy) after extraction in 50:50 (vol/vol) i-butanol/water (Van der Meer et al.), total bilirubin and UCB were measured by HPLC (Spivak and Yuey) after ion-pair extraction with 0.1 mol/L di-n-octylamine acetate in MeOH (McDonagh and Palma), and urobilinogen was quantified by direct spectrophotometry of its zinc complex (Kotal and Fervy). Data given as means ± SEM.

*Statistically significant differences (P < 0.05) vs. control.

Table 5. C57L/J Mice: Fecal Excretion of Bile Salts, Total Bilirubin, UCB, and Urobilinogen After 1 Week on Diet

<table>
<thead>
<tr>
<th>Diet</th>
<th>%</th>
<th>n</th>
<th>Bile salts (µmol/100 g body wt · 24 h−1)</th>
<th>Bilirubin (µmol/100 g body wt · 24 h−1)</th>
<th>UCB (nmol/100 g body wt · 24 h−1)</th>
<th>Urobilinogen (nmol/100 g body wt · 24 h−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>9</td>
<td>0.3 ± 0.03</td>
<td>12.6 ± 2.2</td>
<td>6.4 ± 1.7</td>
<td>176 ± 18</td>
</tr>
<tr>
<td>UDCA</td>
<td>0.25</td>
<td>9</td>
<td>5.1 ± 2.2</td>
<td>4.8 ± 1.4</td>
<td>3.7 ± 1.1</td>
<td>170 ± 80</td>
</tr>
<tr>
<td>CDCA</td>
<td>0.25</td>
<td>9</td>
<td>6.2 ± 0.9</td>
<td>5.9 ± 1.8</td>
<td>4.3 ± 0.8</td>
<td>182 ± 36</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1</td>
<td>9</td>
<td>2.4 ± 0.1</td>
<td>7.8 ± 1.6</td>
<td>6.0 ± 1.4</td>
<td>110 ± 46</td>
</tr>
</tbody>
</table>

NOTE. Bile salts were measured by an enzymatic method (Turley and Dietschy) after extraction in 50:50 (vol/vol) i-butanol/water (Van der Meer et al.); bilirubins were measured by HPLC (Spivak and Yuey) after extraction with 0.1 mol/L di-n-octylamine acetate in MeOH (McDonagh and Palma); urobilinogen was quantified by direct spectrophotometry of its zinc complex (Kotal and Fervy). Data given as means ± SEM.

*Statistically significant differences (P < 0.05) vs. control.
RATS: BILE ACID AND CHOLESTEROL FEEDING EXPERIMENTS

**Bile salt composition**

Bile of chow-fed rats, consisted predominantly of Tβ-MC (42.9 ± 6.7%) and taurocholate (49.5 ± 2.3%) with small amounts of TCDC (5.6 ± 1.%) and TDC (2.0 ± 0.7%). With high dose UDCA, the bile salt pool consisted of 54.7 ± 3.7% TUDC and 32.5 ± 9.4% GUDC with Tβ-MC reduced to 9.8 ± 4.4%, TC to 0.34 ± 0.2% and TCDC to 2.2 ± 1.0%. High dose (2%) CDCA replaced approximately half of the bile salt species with TCDC (22.7 ± 2.4%) and GCDC (25.8 ± 3.3%), whereas the remainder consisted of TUDC (17.3 ± 1.0%), Tβ-MC (20.4 ± 2.4%), Gβ-MC (8.9 ± 0.6%), GUDC (3.6 ± 0.6%) and TC (1.2 ± 0.4%). These changes are reminiscent of bile salt patterns in mouse bile on the bile acid diets (Table 1), however, glycine-conjugated bile salts became more prominent in rats.

**Table 6. Sprague-Dawley Rats: Biliary Lipid Secretion Rates During Acute Biliary "Washout" Performed at 2 Weeks on Diet**

<table>
<thead>
<tr>
<th>Diet</th>
<th>%</th>
<th>n</th>
<th>0-15 min b</th>
<th>0-15 min b</th>
<th>0-15 min b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.04</td>
<td>6</td>
<td>13.6 ± 1.0</td>
<td>0.48 ± 0.06</td>
<td>42 ± 3</td>
</tr>
<tr>
<td>UDCA 0.04</td>
<td>3</td>
<td>17.3 ± 2.7</td>
<td>0.54 ± 0.09</td>
<td>80 ± 2</td>
<td></td>
</tr>
<tr>
<td>UDCA 0.08</td>
<td>3</td>
<td>16.8 ± 3.7</td>
<td>0.53 ± 0.09</td>
<td>77 ± 3</td>
<td></td>
</tr>
<tr>
<td>UDCA 1.00</td>
<td>5</td>
<td>26.3 ± 1.7c</td>
<td>0.59 ± 0.08</td>
<td>45 ± 7</td>
<td></td>
</tr>
<tr>
<td>CDCA 0.04</td>
<td>2</td>
<td>18.4 ± 2.5</td>
<td>0.51 ± 0.15</td>
<td>86 ± 2</td>
<td></td>
</tr>
<tr>
<td>CDCA 0.08</td>
<td>2</td>
<td>14.2 ± 1.2</td>
<td>0.44 ± 0.05</td>
<td>60 ± 7</td>
<td></td>
</tr>
<tr>
<td>CDCA 1.00</td>
<td>2</td>
<td>30.3 ± 9.6c</td>
<td>0.73 ± 0.04</td>
<td>46 ± 7</td>
<td></td>
</tr>
<tr>
<td>CDCA 2.00</td>
<td>2</td>
<td>16.0 ± 1.6</td>
<td>0.53 ± 0.07</td>
<td>26 ± 3</td>
<td></td>
</tr>
</tbody>
</table>

NOTE. Total bile salts and phospholipids in bile were measured by enzymatic methods (Turley and Dietschy29 and Bartlett30) and cholesterol by HPLC (Vercaens et al.26). Data given as means ± SEM.

bDietary additions and percentages administered.

bTiming of biliary washout from insertion of cannula.

bStatistically significant differences (P < 0.05) vs. control.

**Biliary lipid secretion rates**

Table 6 lists biliary lipid secretion rates in rats for the 0-15 "washout" period from time of cannulation at 2 wks of feeding. Bile salt secretion rates increased significantly with 2%
UDCA and 1% CDCA, compared with low dose bile acid and chow-fed controls, but with the highest CDCA dose there is a sharp decrease. In general, phospholipid secretion rates, paralleled bile salt secretion rates, whereas cholesterol secretion rates increased significantly with 0.04% UDCA and are unchanged at higher doses (Table 6).

![Figure 3. Total bilirubin secretion rates (in nanomoles per hour per 100 g body wt) during 0-15 minutes of biliary washout performed after 2 weeks of laboratory chow, udca, or CDCA administration to Spraque-Dawley rats. □, Chow-fed animals; ■ UDCA-fed animals; ■ CDCA-fed animals. *Statistically significant differences ($P < 0.05$) between groups (therefore, total bilirubin secretion rate into bile is significantly greater with 2% UDCA compared with 0% [chow] and 2% CDC. Data given as means ± SEM.

**Biliary bilirubin secretion, plasma unconjugated bilirubin and fecal bilirubin excretion**

Figure 3 displays mean bilirubin secretion rates in bile acid fed rats at 2 wks (from Table 6). The values are increased significantly ($P < 0.05$) by the highest (2%) UDCA dose, but not by CDCA or lower UDCA doses, though there was an upward trend with all increments in bile acid feeding. As found in mice (Fig. 1), bilirubin secretion rates with the lowest (0.04, 0.08%) CDCA levels were comparable to those with the lowest UDCA doses. However, 1% of either bile acid, increased bilirubin secretion rates appreciably compared with controls. In rats given the highest CDCA dose, bilirubin secretion rates were reduced by approximately 20%, but this difference did not reach statistical significance.

With laboratory chow, the plasma UCB levels of rats ($n = 4$) was $1.1 ± 0.3$ (nmol/100g B.W.), bilirubin secretion rate into bile was $29.0 ± 2.3$ (nmol/hr/100g B.W.), and fecal UCB
excretion rates was $13 \pm 2.8$ (nmol/g dry feces). Following 0.5 and 1% cholesterol diets for 2 wks, the higher cholesterol dose increased plasma UCB concentration ($20.4 \pm 3.0$), biliary bilirubin secretion rates ($34 \pm 2.1$) and fecal UCB excretion ($18 \pm 3.0$) significantly, whereas 0.5% cholesterol induced a slight but non-significant increase in serum UCB to $8.0 \pm 1.4$ (nmol/100g B.W.).

**Discussion**

We demonstrated previously that ileectomy in the rat doubled bilirubin secretion rates into bile, and we marshaled experimental evidence to suggest that this was most likely caused by enterohepatic cycling of bilirubin as a consequence of bile salt malabsorption. We now show that orally-ingested unconjugated bile acids, particularly UDCA, and to a lesser extent CDCA, and added dietary cholesterol, resulted in increased secretion of bilirubin into bile attributable to enterohepatic cycling of bilirubin as well as elevating plasma UCB levels in two rodent species.

Animal and human studies have shown that UDCA, most likely in the ionized state as well as its T- and G-conjugates following hepatic conjugation, compete with endogenous bile salts for ileal bile acid transporters (IBAT), leading to bile salt malabsorption. Two percent UDCA has been demonstrated to reduce ileal uptake of cholate conjugates in Sprague-Dawley rats, whereas the same dose of CDCA did not have this effect. The “cholestryamine” effect of dietary cholesterol in rats and humans is explained by a downregulation in IBAT gene expression. We hypothesized that UDCA or cholesterol in the chow diet, by augmenting endogenous bile salt spillage into the cecum, should induce EHC of bilirubin as did ileectomy on rodents. As research models we employed the C57L/J mouse and Sprague-Dawley rat, mainly because of their availability. The inbred mouse is susceptible to cholesterol gallstones when fed a dairy fat-cholesterol-cholic acid diet and is employed as a model to study cholesterol-gallstone (Lith) genes. We found here that a moderate (0.25%) dose of UDCA resulted in a sixfold increase in UCB content of the cecum in mice, whereas in the large intestine, UCB levels remained at control levels (Table 4). This suggests that when ingesting UDCA, UCB that formed in excess was rapidly absorbed from the mouse proximal colon. This interpretation is supported by the observation that total bilirubin and UCB
excretion in feces decreased (Table 5), whereas plasma UCB concentration (Table 4) and bilirubin secretion into bile (Table 3) were increased significantly. Furthermore, UDCA but not CDCA also appeared to enhance urobilinogen formation (Table 4).

We believe that several factors could contribute to an augmented EHC of bilirubin in the setting of increased colonic bile salt levels (Table 6). In UDCA-fed mice, increased cecal bile salt levels may solubilize bilirubin after its deconjugation as evidenced by the marked increase in cecal UCB levels in these rodents (Table 4). Another factor might be that bile salts may increase bacterial β-glucuronidase activity in the distal intestine by enhancing the permeability of bacterial cells. This may promote bilirubin deconjugation, a prerequisite for passive bilirubin transport across the intestinal mucosa. Bile salts may also bind intraluminal calcium as soluble calcium complexes, thereby preventing bilirubin, which has a high affinity for calcium, from complexing the cation to form insoluble calcium salts. All the above factors may help enhance bilirubin levels in solution and promote its intestinal reabsorption. Nonetheless, UDCA and CDCA produced comparable increases in cecal bile salts, no doubt contributed primarily by the ingested species, yet UDCA had a distinct effect on increasing cecal UCB content and decreasing this level in the colon. In other work we have shown that conjugated UDCA has a protective effect on bilirubin oxidation and may promote UCB absorption through incorporation of the molecule on bile salt monomers and micelles.

In mice fed a 0.25% UDCA, gallbladder bile revealed a significant increase in UCB concentration (Table 2), and precipitates of calcium bilirubinate were observed in two thirds of gallbladder bile. This indicates that in UDCA-fed animals, the solubility product of the acid (or less likely neutral) salt of calcium and UCB in gallbladder bile was exceeded, possibly augmented by the lower solubility of UCB at neutral pH in mixed TUDC-rich bile salt micelles. Clearly, it would be of interest to determine if UDCA, at this modest level, could induce pigment gallstone formation in mice with more prolonged feeding.

In the current work, we fed unconjugated bile acids of differing hydrophilic-hydrophobic balance, which are absorbed by passive diffusion throughout the small intestine and subsequently extracted and conjugated efficiently by the liver and re-secreted into bile. Following high dose UDCA in mice, the bile salt species was replaced almost 90% by TUDC (Table 1), whereas in rats, the bile salt pool consisted of the same total percentage of T- and
G-conjugates of UDCA (55 and 33%, respectively, see Results). Therefore, we predicted that ileal uptake of endogenous bile salts would decrease, and as a result, endogenous bile salt malabsorption would occur. This is consistent with our findings that compared with chow-fed controls, fecal bile salt excretion (Table 5) increased 18-fold, and bile salt levels in the large intestine of mice increased almost fourfold (Table 6). Bile salts in the proximal large intestine of mice fed high-dose CDCA increased by the same order of magnitude (Table 4), as expected for the large exogenous intake, yet the rates of bilirubin secretion into bile, although elevated, were not significantly different from controls, suggesting a less extensive EHC of bilirubin with CDCA. This is supported by the observation that only moderate increases of serum UCB concentrations occurred in these animals due to the low (approximately 30%) first-pass hepatic extraction of UCB. Following 0.5% CDCA, biliary bile salt composition of mice shifted to mainly hydrophobic bile salts, especially TCDC. A possible explanation for an absent enterohepatic cycling of unconjugated bilirubin under these conditions, is that these hydrophobic bile salts have bactericidal effects, possibly preventing or delaying bilirubin deconjugation. Further, hydrophobic bile salts, by causing bacterial cell lysis, may decrease β-glucuronidase activity in the intestinal tract. Moreover, since extensive biotransformation of low-dose CDCA to TMC's occurred (Table 1), these bile salts, which are even more hydrophilic than TUDC could have promoted enterohepatic cycling of bilirubin and its secretion into bile (Table 5), thus mirroring the effects of UDCA. A contributing explanation, which needs further exploration, is whether colonic bile salts may influenced enterocyctic metabolism of protoporphyrin or heme, which could be metabolized in the intestinal wall to UCB.

In studies with Syrian hamsters, Dam and colleagues demonstrated that 1% cholesterol added to a diet containing 10% butter fat lowered the incidence of cholesterol gallstones, but increased that of pigment gallstones. This was further supported by LaMorte and associates, who found that 100% of female Hartley guinea pigs fed a 0.5% cholesterol-supplemented diet developed pigment gallstones after 12 weeks. Along these lines, Strichartz and colleagues showed that total bilirubin and UCB levels in gallbladder bile of prairie dogs increased two- to threefold on a 1.2% cholesterol-enriched diet. Consistent with the hypothesis that dietary cholesterol would induce spillage of bile salts into the large intestine in excess and induce EHC of bilirubin, is our observation that 1% cholesterol in the diets of mice significantly
increased cecal bile salt and UCB levels, as well as plasma UCB concentrations and biliary bilirubin secretion rates (Fig. 2, Tables 4, 5). However, further increases in dietary cholesterol reduced bilirubin secretion rates into bile (Fig. 2). Although the reason for this observation is not clear, it is possible that cholesterol and bilirubin molecules may interfere with each other’s absorption, conjugation or secretion, in the intestinal tract or liver. Of interest is that despite higher bilirubin secretion rates into hepatic bile (Fig. 2), the concentrations of bilirubin and UCB in gallbladder bile of mice fed cholesterol (Table 2) were similar to animals fed laboratory chow, suggesting that a lower fraction of bile flow may have entered the gallbladder, possibly because of gallbladder dysmotility, a known toxic effect of cholesterol on smooth muscle contraction.\textsuperscript{50}

In rats, 1% dietary cholesterol produced almost a threefold increase in serum UCB concentration, which was associated with a significant increase in biliary bilirubin secretion rate (see Results). This shows that dietary cholesterol also resulted in EHC of bilirubin in rats, and this may be coupled with bile salt inhibition of bilirubin’s catabolism to urobilinogen for the reasons discussed, and perhaps bile salt-induced hypermotility of the gut which may explain why UCB excretion in feces was significantly increased in these animals (see Results).

The cholestyramine effect\textsuperscript{19,20} of 1% cholesterol in mice was exemplified by an eightfold increase in bile salt loss in feces (Table 5), confirmed by the fourfold increase of bile salt levels in the large intestine (Table 4). This is consistent with increased small intestinal absorption of cholesterol molecules downregulating transcription of the IBAT gene.\textsuperscript{21} Furthermore, the bile salt pool in these animals was relatively enriched in TMC’s, and should therefore like UDCA conjugates,\textsuperscript{48,51} compete with endogenous bile salts for absorption via IBAT. TUDC, when fed to humans to effect gallstone dissolution, induces stone calcification as frequently as UDCA,\textsuperscript{52} suggesting that EHC of bilirubin may indeed be a normal phenomenon in mice, especially because of high endogenous TMC levels.

Not unexpectedly, bile acid feeding increased total biliary bile salt secretion rates in both rodent species. The relatively small difference in bile salt secretion rates between UDCA doses (Table 6) paralleled fecal losses. With the highest CDCA dose, all biliary lipid secretion rates in rats fell (Table 6), suggesting some degree of bile acid toxicity since 0.25% CDCA caused a moderate increase in serum alanine aminotransferase and alkaline phosphatase
activities (see Results). We did not observe a similar pattern in mice (Table 3), possibly because the high TMC levels in bile protected liver function against hydrophobic bile salt injury.\textsuperscript{54}

In summary, we have demonstrated that bilirubin secretion rates into bile of rodents were increased significantly by chow diets supplemented with UDCA and cholesterol. This is caused most likely by endogenous bile salt malabsorption, which apparently results in enterohepatic cycling of bilirubin from the cecum and large intestine. This concept is bolstered by elevated serum concentrations of UCB and intestinal levels of urobilinogen, as well as differences in their levels between large intestine and feces. These combined studies in two rodent models are internally consistent although further studies will be necessary to provide more direct, i.e., radiotracer evidence, for enterohepatic cycling of bilirubin. Therefore, we believe that in addition to ileectomy as an etiology for enterohepatic cycling of bilirubin in rodents, this work expands the concept by identifying putative chemical/dietary causes related to the function of the ileal bile acid transporter. If our observations are applicable to humans, then they might provide explanations for several clinical observations. For example, the frequent failure of UDCA to dissolve cholesterol gallstones\textsuperscript{12} is often associated with calcification and "pigment shell" formation on these stones.\textsuperscript{7-14} Serum bilirubin levels may increase mildly in response to UDCA administration and this may reflect enterohepatic cycling of bilirubin rather than inhibition of conjugation at the hepatocellular level.\textsuperscript{55} Extrapolating from the current rodents studies to humans, we aver that long term UDCA therapy might elevate biliary levels of conjugated bilirubins, possibly UCB or even formation of pigmentary "biliary sludge." It is tempting to speculate that enterohepatic cycling of bilirubin from ingestion of large "boluses" of dietary cholesterol may be related to the calcium bilirubinate nidus invariably found in the purest cholesterol gallstones.

\textbf{References}


43. Ostrow JD, Celic L. Bilirubin chemistry, ionization and solubilization by bile salts. Hepatology 1984;4:38S-45S.


Acknowledgements. The authors thank Monika Leonard and Dr. David Q. Wang for their superb technical assistance and Pelle Cass for word processing the manuscript.