Pathogenesis of gallstones in Crohn's disease
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Chapter 3

BILIRUBIN CYCLES ENTEROHEPATICALLY AFTER ILEAL RESECTION IN THE RAT
Background & Aims: Patients with ileal disease, resection or bypass are at increased risk of developing pigment gallstones, but the pathophysiological mechanisms are unknown. The aim of this study was to test the hypothesis that ileectomy induces enterohepatic cycling of bilirubin.

Methods: Ileectomy or sham operation was performed in adult male Sprague-Dawley rats with the following control procedures: no operation, ileal transection, proximal or distal jejunectomy, ileocolonic transposition and ileocecectomy. Bilirubin and bile salt secretion rates were measured after bile duct cannulation performed 3-11 days after intestinal surgery. Also measured were bilirubin and bile salt concentrations in the colon as well as indices of hemolysis in blood.

Results: Compared with controls, bilirubin secretion rates were increased significantly 3-5 days after ileectomy, distal jejunectomy, ileocolonic transposition, and ileocecectomy, with no hemolysis occurring. Bile salt secretion rates also increased significantly after ileectomy but decreased markedly with prevention of coprophagy, whereas bilirubin secretion rates remained elevated. By 8-11 days after surgery, intestinal adaptation normalized bile salt reabsorption, and hypersecretion of bilirubin was abolished. Colonic levels of unconjugated bilirubin and bile salts were increased fivefold and eightfold respectively in ileectomized animals, but unconjugated bilirubin levels remained normal in bile.

Conclusions: These results are consistent with the hypothesis that enterohepatic cycling of bilirubin occurs with bile salt malabsorption.
Introduction

Patients with ileal disease, resection or bypass (e.g., Crohn's disease, surgery for morbid obesity, or hypercholesterolemia) are at increased risk for developing gallstone disease, whereas prevalence of cholelithiasis in patients with ulcerative colitis is comparable with the prevalence in the general population. Hitherto it was believed that ileal dysfunction, which interrupts the enterohepatic circulation (EHC) of bile salts, resulted in cholesterol supersaturation of bile and cholesterol gallstone formation. Short-term biliary diversion may augment cholesterol saturation of bile, particularly in patients with preexisting defects in biliary lipid secretion; nonetheless, studies in both humans and rhesus monkeys have indicated that cholesterol supersaturation of bile is not sustained. Presumably, this occurs because of marked derepression of hepatic cholesterol \( \Delta\alpha \)-hydroxylase activity, the rate limiting enzyme in conversion of cholesterol to bile salts, with the result that more hepatic free cholesterol is channeled into de novo bile salt synthesis. This concept is consistent with observations on bile of patients with Crohn's disease, showing that they are either unsaturated or significantly less saturated with cholesterol than in controls.

In the ileectomized prairie dog, a cholesterol-rich diet leads to development of lithogenic bile and cholesterol gallstones, whereas a trace-cholesterol diet induces pigment gallstone formation. Microscopy and analysis of prairie dog bile after ileal resection showed calcium bilirubinate precipitates and small pigment gallstones, as well as increased levels of calcium and total bilirubin, but normal cholesterol saturation indexes. Furthermore, Coyle et al. showed that ileal bypass in the guinea pig also resulted in calcium bilirubinate gallstone formation. On the basis of these experiments, Pitt et al. speculated that gallbladder stasis, hormonal changes, gallbladder mucus production, and hemolysis may play a role in pigment precipitation, but insights into possible pathophysiological mechanisms were lacking. Although in the prototypical study of humans with ileal disease the investigators noted that the majority of the gallstones were radiopaque and, therefore, rich in inorganic calcium salts, so far only one group has verified by stone analysis that the prevalence of pigment as opposed to cholesterol gallstones is increased in patients with Crohn's disease. Consistent with this concept are recent findings during surgery that both conjugated and unconjugated bilirubin (UCB) levels are elevated markedly in gallbladder bile of patients with Crohn's disease involving the ileum.
Another common complication in patients with ileal dysfunction is calcium oxalate urolithiasis, which is caused by enteric hyperoxaluria. Chadwick et al. proposed that, with fat malabsorption, intraluminal calcium is precipitated as calcium soaps and that this results in increased levels of oxalate in solution with subsequent enhanced absorption from the intestine. To induce the enteric hyperoxaluric syndrome, bile salts and/or fatty acid spillage as well as an intact colon seem to be necessary, suggesting that altered mucosal permeability of the colon to the dianion is crucial. Because both monocarboxylic or dicarboxylic oxalate (with $pK_a$ of $\text{H}_2\text{O}$ = 1.3 and 4.3 at 25°C) and bilirubinate (proposed $pK_a$ of 0.15 mol/l NaCl) ≈ 6.8/8.1 and 8.4/9.3 at 21°C-25°C have high affinities for calcium, we hypothesized that enteric hyperbilirubinia may be the endogenous equivalent of enteric hyperoxaluria in patients with bile salt malabsorption. To test this hypothesis, we investigated whether ileectomy-induced bile salt malabsorption in the rat resulted in increased bilirubin secretion into bile. The results are consistent with our postulate and suggest that an intestinal deconjugation, absorption, hepatic extraction, and reconjugation cycle occurs for bilirubin in ileal disease, just as is well established for bile salt molecules in the healthy state. This provides a putative pathophysiological mechanism for increased bilirubin conjugate levels in bile of patients with bile salt malabsorption.

**Materials and Methods**

**Animals**

Male Sprague-Dawley rats (Charles River Breeding Laboratories Inc., Wilmington, MA), 200-300 g body wt, were studied. Animals were maintained in cages at 23°C with a 12-hour daylight cycle and were fed standard rat chow. All aspects of the study conformed to accepted criteria for the care and experimental use of laboratory animals, and were consistent with euthanasia recommendations of the American Veterinary Medical Association. Protocols were approved by the Harvard Medical Area Standing Committee on Animals.

**Surgical Procedures**

Rats were fasted for 36 hours and, after pentobarbital anesthesia (35 mg/kg body wt intraperitoneally), surgery was performed under sterile conditions. After delivery through a
midline abdominal incision, the small and large intestines were placed on hot-moistened gauzes. For ileectomy or distal jejunectomy, 8-cm segments of intestine 0.5 cm or 16.5 cm proximal to the ileocecal junction, respectively, were resected. Intestinal continuity was restored with end-to-end anastomoses performed with a layer of 5-0 silk sutures. In the ileoceleocrectomy group, resection was followed by an end-to-end jejunocolonic anastomosis. For ileocolonic transposition, the ileum was mobilized and divided, leaving vascular supply and innervation intact. The ileal segment was then interposed in a transected proximal colon, 5 cm distal to the cecocolonic junction. For proximal jejunectomy, 8 cm of small bowel was resected 2 cm distal to the ligament of Treitz, and the severed ends were reanastomosed. In the ileal transected group, the bowel was divided 0.5 cm proximal to the ileocecal junction and then was reanastomosed. Sham operation consisted of an exploratory laparotomy with mobilization of the small bowel. By definition, unoperated animals did not undergo surgery, but their bile ducts were cannulated later to measure biliary bilirubin and lipid secretion rates. Body temperature (37°C) was maintained during surgery and for 24 hours thereafter using a heating lamp and monitored with a rectal probe. At termination of surgery, the abdominal cavity was rinsed with warm sterile saline, and the abdominal wall was closed with 3-0 silk sutures; gentamycin (7 mg/kg body wt) was then administered intramuscularly. Postoperatively, animals were allowed free access to water, but food was withheld for the first 36 hours.

**Experimental Design**

Before and after surgery, stool appearance and body weight were recorded daily. In all animal groups, 3 days after surgery, short-term biliary washout studies were performed after bile duct cannulation; in other sets of ileectomized, ileocolonic transpositioned, and sham-operated animals, similar studies were performed at 3, 5, 8 and 11 days after surgery. Because the jejunum plays an important role in maintaining the EHC of bile salts in the rat, distal as well proximal jejunectomies were performed. Furthermore, because rapid adaptation of bile salt reabsorption in the remaining small intestine of the rat can restore EHC of bile salts to normal quickly, we constructed an ileocolonic transposition to establish permanent bile salt spillage into the proximal colon without fecal bile salt loss. Ileocecectomy was performed to examine whether removal of the cecum would prevent EHC of bilirubin. In humans, the cecum is more
acidic (pH 5-6) than the remainder of the large bowel\textsuperscript{31} and, therefore, potentially a favorable site for absorption of the diacidic UCB.

Because rats are coprophagous, the possibility existed that changes in biliary bile salt and bilirubin secretion rates may reflect an EHC of bile salts via the fecal-oral route. Therefore, in a subset of ileectomized as well as ileal-transected animals, coprophagy was prevented effectively throughout the experiment period by using a coarse-mesh screen-floor cage and a permanent 4-5-cm-wide cerival collar of stiff plastic. Two days before abdominal surgery and after short-term ether anesthesia, the collar was secured circumferentially to the cervical skin with four (2-0) sutures.

To establish that conversions of UCB and bile salt in the large intestine were consistent with biliary lipid secretion rates during bile collection, the cecum as well as 5 cm of proximal colon were resected in four groups of ileectomized and ileal-transected animals on the third postsurgery day. For subsequent analysis, cecal and proximal large bowel contents were frozen rapidly, freeze-dried, and stored at -70°C. Blood was obtained from the same animals by needle puncture of the inferior vena cava and analyzed immediately for hemolytic indices including hematocrit, hemoglobulin level, and reticulocyte count, as well as total bilirubin levels.

\textbf{Cannulation of the Bile Duct and Biliary Washout}

Under light pentobarbital anaesthesia, surgery was performed on nonfasted animals between 9 and 11 AM. After a midline abdominal incision, the entire small and large intestines were placed on moist gauzes and examined grossly for macroscopic hypertrophy. The bile duct was then cannulated with a PE-10 polyethylene catheter with an ID of 0.28 mm, and an OD of 0.61 mm. The catheter was sufficiently large to prevent acute cholestasis and suitably short to minimize dead space. Using a fraction collector, bile was collected in tared tubes at 15-minute intervals for the first 0.5 hour, and at 30-minute intervals for the next 1.5 hours. All tubing and glassware were covered with aluminium foil, and lights were dimmed to protect bilirubin from photodegradation. After the biliary catheter was sutured to the abdominal wall, the abdomen was closed, and animals were placed in a restraining cage. Water was supplied ad libitum, and each animal’s body temperature was maintained at 37°C as described previously. Bile volume was determined gravimetrically, assuming a specific gravity of unity, and samples were stored on ice under argon in the dark for <2 hours. Fresh bile was also analyzed for bile pigments by high-
performance liquid chromatography and for calcium bilirubinate precipitates using direct and polarized light microscopy. Bile was then frozen and stored at -20°C for later analysis of biliary lipids. Total bile salt and bilirubin secretion rates were calculated for washout intervals of up to 2 hours by multiplying biliary concentrations by volumes. Bilirubin diconjugate/monoconjugate ratios were calculated by dividing the concentrations of bilirubin diglucuronide (BDG) plus bilirubin monoglucuronide-glucoside (BMGGI) by the concentration of bilirubin monoglucuronide (BMG). Total biliary calcium was measured in 5 sham-operated and 5 ileectomized animals with and without prevention of coprophagy. Immediately after experimentation, all animals were killed with an overdose of diethyl ether or pentobarbital.

**Analytical Methods**

Molecular species of bilirubins were separated and quantified according to the high-performance liquid chromatography procedures of Spivak and Yuey, and total bile salts were assayed by the 3α-hydroxysteroid dehydrogenase method. Biliary phospholipids were measured with Bartlett’s assay, and biliary cholesterol was measured by high-performance liquid chromatography. Total calcium was determined by atomic absorption spectrometry. Cecal and proximal colonic bilirubins were extracted with 0.1 mol/L of the ion-pairing agent di-n-octylamine acetate in MeOH from 10- to 50-mg portions of dry large intestinal contents and analyzed by high-performance liquid chromatography. From another portion of colonic contents, bile salts were extracted with t-butanol/water (50:50, vol/vol) as described by Van der Meer et al. and were measured enzymatically.

**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). Student’s t test was used for comparing bile secretory data in ileectomized and sham-operated animals 3-11 days after surgery, as well as for comparison of washout data of ileectomized and ileal transected animals with concomitant prevention of coprophagy.
Results

Animal Weight and Stool Appearance

Body weights of ileal-resected, proximal and distal jejunectomized, ileocolonic-transpositioned, and ileocecectomized animals decreased ≤10% during the first 5 days after-surgery. In contrast, by the third postoperative day, body weights of sham-operated and ileal-transected animal increased significantly (P < 0.005) compared with ileocecectomized animals. By 8 and 11 days after-surgery, body weights of animals (230-375 g) were not significantly different from nonoperated controls (230-440g). Transient (2-3 days) diarrhea, occurred in 22 of 26 ileectomized (81%), 3 of 6 ileocecectomized (50%), 2 of 6 distal jejunectomized (33%), 2 of 12 ileal-transected (17%) and 1 of 6 ileocolonic-transpositioned animals (17%). None of the sham-operated or unoperated animals or those with proximal jejunectomy developed diarrhea. All animals recovered rapidly from surgery and, despite self-limiting weight loss and diarrhea, were otherwise healthy throughout the experiments.

Intestinal Adaptation

As reported by others on the basis of histological criteria,38 hypertrophy of the residual intestine followed distal small bowel resection in the rat and was evident on the third postsurgery day. Both ileectomy as well as distal jejunectomy resulted in hypertrophy of remaining distal small bowel and cecum.39 Ileocecectomized animals developed hypertrophy of the jejunum as well as the proximal colon. Hypertrophy of the distal small bowel followed proximal jejunectomy as noted by others.40,41 In animals with ileocolonic-transposition, intestinal hypertrophy was confined mainly to the proximal colon and transpositioned ileum. As expected, sham-operated animals did not develop intestinal hypertrophy, but ileal-transected animals developed modest bowel enlargement without evident obstruction in the vicinity of the anastomosis.

Biliary Lipid, Bilirubin, and Calcium Secretion Rates

Figure 1 shows representative high-performance liquid chromatography profiles of bilirubins in bile of sham and small bowel-resected animals at 3 days after surgery and collected during the earliest (0-15 minutes) biliary washout period. Figure 1A shows the chromatogram of
bile from a sham-operated animal that contained almost equimolar amounts of BDG and the two isomers of BMG, whereas the concentration of BMGGI (peak no. 3) was <3 % of the total. Figure 1B, and C shows bilirubin chromatograms in biles of ileectomized animals, where two distinct patterns were observed. In Figure 1B, biliary BDG was elevated, whereas BMG levels were unchanged. However, in Figure 1C, relatively low BDG and BMG levels were accompanied by markedly elevated BMGGI levels. Figure 1D shows the chromatogram for bilirubins of a distal jejunectomized animal, showing a pattern similar to that in Figure 1C, except that BMG levels were increased and BMGGI levels were less elevated. In all chromatograms, UCB concentration (peak no. 5) were either nonexistent or <1% of total bilirubins.

Figure 1. Elution patterns of bile pigments in rat bile (absorbance at 450 nm) by high-performance liquid chromatography during 0-15 minutes of a biliary washout performed 3 days after (A) sham laparotomy, (B and C) ileectomy, and (D) distal jejunectomy. Methods and identification of peaks were performed according to the procedures of Spivak and Yuey.1, solvent peak; 2, BDG; 3, BMGGI (peak no.3) were observed in about half of the (C) ileectomized and (D) distal jejunectomized animals. Increased BMG levels (peak no.4) were highly variable. Note differences in ordinate scales between A and C and B and D.

Figure 2A plots mean (± SEM) of total bilirubin secretion rates in bile during 0-30 minutes of biliary washout performed 3 days after surgery. Bilirubin secretion rates were increased significantly \( (P < 0.001) \) after ileectomy, distal jejunectomy, ileocolonic transposition, and ileocecectomy compared with proximal jejunectomized, unoperated, sham and ileal-transected controls. Figure 2B shows mean diconjugated/monoconjugated bilirubin ratios in bile of the same animals. The diconjugate/monoconjugate ratio was increased significantly \( (P = 0.001) \) after distal jejunectomy compared with proximal jejunectomized and unoperated animals. Although bilirubin diconjugate/monoconjugate ratios (Figure 2B) in bile were increased after
ileectomy, ileocolonic-transposition, and ileoceccectomy compared with controls, they failed to reach statistical significance. Figure 2C shows total bile salt secretion rates for the same conditions. Bile salt secretion rates increased significantly ($P = 0.005$) after ileectomy compared with sham-operated animals; and all other groups gave intermediate and nonsignificant values. Bile salt secretion rate was decreased approximately 50% in sham-operated controls, but this difference was not statistically significant compared with ileal-transected and unoperated animals (Figure 2C). Biliary phospholipid outputs in sham-operated animals were $1.0 \pm 0.2$ umol/h/100g compared with $2.2 \pm 0.3$ and $2.2 \pm 0.2$ umol/h/100g, respectively ($P < 0.001$), in ileectomized and ileal-transected animals. For the same conditions, biliary cholesterol outputs were $0.11 \pm 0.01$ vs. $0.22 \pm 0.05$ and $0.17 \pm 0.02$ umol/h/100g ($P < 0.01$), respectively, and the outputs of both lipids paralleled bile salt secretion rates (Figure 2C). Secretion rates of biliary calcium in ileectomized rats ($3.0 \pm 0.4$ umol/h/100g) were not significantly different from sham-operated animals ($2.6 \pm 0.3$ umol/h/100g).

Figure 2. Secretion rates of bilirubin and bile salts during 0-30 minutes of biliary washout performed 3 days after intestinal surgery in all animal groups ($n = 5-9$ for each). (A) Total bilirubin secretion rate (in nanomoles per hour per 100 g body wt). (B) Bilirubin diconjugate / monocojugate ratio. (C) Total bile salt secretion rate (in micromoles per hour per 100 g body wt). *Statistically significant differences ($P < 0.005$) between perpendicular serifs within and at ends of the square backets. Data are expressed as means ± SEM.
Table 1 summarizes total bilirubin secretion rates during biliary washout in all groups 3 days after intestinal surgery. During the initial washout period (0-30 minutes), bilirubin secretion rates were increased significantly in animals with distal small bowel resections, including ileocolonic transposition. However, during subsequent 30-60 and 60-90 minutes of washout, total bilirubin secretion rates decreased in all animals, and comparisons between experimental and control groups became less significant or were not different. By 90-120 minutes of biliary diversion, bilirubin secretion rates in ileocolonic-transpositioned and ileectomized rats were identical with those during the earliest times of the washout (Table 1).

Table 1. Total Bilirubin Secretion Rates in Rats During Biliary Washout Performed 3 Days After Intestinal Surgery

<table>
<thead>
<tr>
<th>Operative group</th>
<th>n</th>
<th>0-30 min</th>
<th>30-60 min</th>
<th>60-90 min</th>
<th>90-120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unoperated</td>
<td>6</td>
<td>23.4 ± 1.9</td>
<td>19.5 ± 1.3</td>
<td>18.0 ± 1.9</td>
<td>23.1 ± 1.8</td>
</tr>
<tr>
<td>Sham</td>
<td>9</td>
<td>26.8 ± 2.0</td>
<td>24.6 ± 1.6</td>
<td>25.1 ± 1.2</td>
<td>29.3 ± 1.7</td>
</tr>
<tr>
<td>Ileal transection</td>
<td>6</td>
<td>29.4 ± 3.0</td>
<td>27.0 ± 1.7</td>
<td>22.4 ± 2.0</td>
<td>25.6 ± 5.3</td>
</tr>
<tr>
<td>Distal jejunectomy</td>
<td>6</td>
<td>32.3 ± 3.7</td>
<td>34.7 ± 3.7</td>
<td>33.6 ± 5.6</td>
<td>39.4 ± 7.9</td>
</tr>
<tr>
<td>Ileocolonic transposition</td>
<td>5</td>
<td>53.1 ± 8.6</td>
<td>43.2 ± 4.1</td>
<td>41.2 ± 3.2</td>
<td>45.8 ± 4.5</td>
</tr>
<tr>
<td>Ileectomy</td>
<td>6</td>
<td>49.7 ± 6.8</td>
<td>34.8 ± 3.6</td>
<td>34.1 ± 5.2</td>
<td>36.8 ± 3.8</td>
</tr>
</tbody>
</table>

NOTE. Data are expressed as means ± SEM.

*Biliary bile salt secretion rates are listed in Table 2. As expected, all bile salt secretion rates were maximal during 0-30 minutes of washout and, over subsequent time intervals, decreased progressively, reaching low values at 90-120 minutes. These were significantly (P < 0.05) lower (40-70%) in unoperated, sham, ileal-transected, and ileocolonic transpositioned animals compared with initial (0-30 minutes) levels. This well-known depletion of the bile salt pool is consistent with complete interruption of the EHC and was reflected in diminished volumes of bile flow (data not shown).
## Table 2. Total Bile Salt Secretion Rates During Biliary Washout Performed 3 Days After Intestinal Surgery

<table>
<thead>
<tr>
<th>Operative group</th>
<th>n</th>
<th>0–30 min $^a$</th>
<th>30–60 min $^a$</th>
<th>60–90 min $^a$</th>
<th>90–120 min $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unoperated</td>
<td>6</td>
<td>11.3 ± 0.8</td>
<td>7.0 ± 0.8</td>
<td>5.6 ± 0.5</td>
<td>5.6 ± 0.9</td>
</tr>
<tr>
<td>Sham</td>
<td>9</td>
<td>6.3 ± 1.0</td>
<td>4.2 ± 0.7</td>
<td>3.1 ± 0.5</td>
<td>3.6 ± 0.8</td>
</tr>
<tr>
<td>Ileal transection</td>
<td>6</td>
<td>13.0 ± 1.7</td>
<td>9.3 ± 2.1</td>
<td>5.9 ± 1.0</td>
<td>5.5 ± 0.9</td>
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<tr>
<td>Proximal jejunectomy</td>
<td>6</td>
<td>11.4 ± 3.0</td>
<td>8.4 ± 1.5</td>
<td>6.8 ± 1.4</td>
<td>7.5 ± 2.1</td>
</tr>
<tr>
<td>Distal jejunectomy</td>
<td>6</td>
<td>12.0 ± 1.8</td>
<td>13.4 ± 3.7$^a$</td>
<td>12.1 ± 4.7</td>
<td>8.9 ± 1.7</td>
</tr>
<tr>
<td>Ileocolonic transposition</td>
<td>5</td>
<td>13.3 ± 0.7</td>
<td>10.6 ± 0.8</td>
<td>8.2 ± 1.0$^a$</td>
<td>9.9 ± 0.8$^a$</td>
</tr>
<tr>
<td>Ileocectomy</td>
<td>6</td>
<td>14.5 ± 2.3</td>
<td>9.7 ± 1.5</td>
<td>9.3 ± 1.2$^a$</td>
<td>8.7 ± 1.4$^a$</td>
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<tr>
<td>Ileectomy</td>
<td>8</td>
<td>17.7 ± 2.8$^e$</td>
<td>12.7 ± 2.5$^e$</td>
<td>9.8 ± 1.7$^e$</td>
<td>10.3 ± 1.7$^e$</td>
</tr>
</tbody>
</table>

**NOTE.** All data are expressed as means ± SEM.

$^a$Total bile salts were measured by an enzymatic Method. $^{31}$

$^b$Surgical preparation of rats as described in Materials and Methods.

$^c$Interval of biliary washout from the time of cannulation of bile duct.

$^d$Statistically significant differences ($P < 0.05$) between resected and/or transpositioned vs. sham-operated animals.

### Surgery

Figure 3A-C summarizes outputs of bilirubin, its diconjugate/monoconjugate ratio, and bile salt secretion rates during 0-120 minutes of biliary washout in ileectomized and sham-operated animals as functions of the number of days after intestinal surgery. Figure 3A shows that, after ileectomy, total bilirubin secretion rate declined progressively from a high level at 3 days to that of the sham-operated animals by the 11th postoperative day, whereas in sham-operated animals, the bilirubin level remained at a constant value from 3 to 11 days. In ileocolonic-transpositioned animals, total bilirubin secretion rates after surgery were comparable to those of ileectomized animals (data not shown). Figure 3B shows that the bilirubin diconjugate/monoconjugate ratios were variable and were not statistically different between ileectomized and sham-operated animals. Figure 3C shows that in ileectomized animals, total bile salt secretion rates were significantly elevated on the third post-surgical day and thereafter decreased appreciably, becoming equivalent to those of the sham-operated animals between 5 and 11 days. In contrast, Figure 3C shows that total bile salt secretion rates in sham-operated animals were significantly lower at 3 days compared with 5-11 days after surgery. In ileocolonic-transpositioned animals, bile salt secretion rates after surgery (data not shown) showed the same behavior as in ileectomized animals (Figure 3C).
Ileal Resection in the Rat

Figure 3. Secretion rates of total bilirubin and bile salts during 0-120 minutes of biliary washout in ilectomized and sham-operated animals as function of the number of postoperative days after intestinal surgery. (A) Total bilirubin secretion rate (in nanomoles per hour per 100 g body wt). (B) Bilirubin diconjugate / monoconjugate ratio. (C) Total bile salt secretion rate (in micromoles per hour per 100 g body wt). ■, ileectomized animals; □, sham-operated animals. Asterisks indicate statistically significant differences between groups: *P < 0.005; **P < 0.05 as indicated by sherifs and bracket ends. Data are expressed as means ± SEM.

Influence of Coprophagy on Biliary Bile Salt, Bilirubin, and Calcium Secretion Rates

Figure 4 shows effects of coprophagy on biliary bilirubin, diconjugate/monoconjugate ratios, and bile salt secretion rates in ileectomized and ileal-transected animals at 3 days postsurgery. Figure 4A shows that total bilirubin secretion rates were significantly elevated (P < 0.005) after ileectomy compared with ileal-transected animals (see Figure 2), and that prevention of coprophagy (open bars) did not influence these values appreciably. In contrast, prevention of coprophagy (Figure 4B) decreased mean bilirubin diconjugate/monoconjugate ratios in both animal groups, and the differences reached statistical significance (P < 0.05) in ileal-transected animals. This influence of coprophagy on the diconjugate/monoconjugate ratio was principally due to biliary BMGGI levels, which were markedly elevated in the coprophagous state (Figure 1C) and diminished to values comparable with those in control animals after its prevention (e.g., Figure 1A). Furthermore, Figure 4C shows that total bile salt secretion rates in ileectomized animals decreased markedly (P < 0.01) with prevention of coprophagy and resulted in biliary bile salt secretion outputs significantly lower (P < 0.05) than in ileal-transected animals. This finding indicates that in the ileectomized state, coprophagous animals were ingesting bile salts via the fecal-oral route and that this contributed significantly to their EHC. Prevention of
coprophagy in ileectomized animals did not influence biliary calcium secretion rates appreciably, showing nonsignificant variations from 2.6 ± 0.1 to 3.0 ± 0.4 μmol/h/100g (body wt).

**Figure 4.** Biliary bilirubin and bile salt secretion rates during 0-30 minutes of biliary washout 3 days after ileectomy and ileal transection in rats with and without prevention of coprophagy. □, rats capable of coprophagy; □, rats in which coprophagy was effectively prevented. (A) Total bilirubin secretion rate (in nanomoles per hour per 100 g body wt). (B) Bilirubin diconjugate / monoconjugate ratio. (C) Total bile salt secretion rate (in micromoles per hour per 100 g body wt). Asterisks indicate statistically significant differences between groups: *P < 0.005; **P < 0.05 as indicated by the ends of the brackets.

**Serum Hemogram Analyses**

In blood drawn at 3 days postsurgery from ileectomized (n = 4) and ileal-transected (n = 3) animals, hemoglobin (13.5 ± 0.8 vs. 14.1 ± 0.9 g/dL), hematocrit (41.9% ± 1.9% vs. 43.65 ± 2.5%), and reticulocyte count (2.7% ± 0.4% vs. 2.6% ± 0.4%) were not statistically different (P > 0.6). Furthermore, plasma total bilirubin levels were below the detectable limit (0.1mg/dL) in both sham and ileectomized animals (n = 2).

**Cecal and Proximal Colonic Bilirubin and Bile Salt Concentrations**

Table 3 lists cecal plus proximal large intestinal bile salt and UCB concentrations in the ileectomized and ileal-transected rats (n = 4 each) at 3 days after intestinal surgery. In ileectomized animals, colonic concentrations of UCB and bile salts were increased fivefold and
eightfold, respectively, compared with ileal-transected rats. Because biliary bilirubin outputs were elevated maximally on the third postoperative day (Table 1), it is likely that these levels approximated the highest intraluminal values.

### Table 3. Colonic Unconjugated Bilirubin and Bile Salt Concentrations 3 Days After Intestinal Surgery

<table>
<thead>
<tr>
<th></th>
<th>Ileectomy (n = 4)</th>
<th>Ileal transection (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unconjugated bilirubin (nmol/g dry intestinal content)*</td>
<td>70.4 ± 32.3†</td>
<td>14.1 ± 2.9</td>
</tr>
<tr>
<td>Total bile salt (µmol/g dry intestinal content)†</td>
<td>35.7 ± 6.7</td>
<td>4.7 ± 1.7</td>
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</table>

NOTE. Data are expressed as means ± SEM.  
*Measured as described in Materials and Methods.  
†Cecal plus proximal large intestinal bilirubins and bile salts were extracted from 10 to 50 mg dry large intestinal contents.  
⁷P < 0.05 between ileectomized and ileal-transected animals.  
⁸P < 0.005 between ileectomized and ileal-transected animals.

### Direct and Polarizing Light Microscopy

At 3 days after surgery, neither calcium bilirubinate precipitates nor cholesterol monohydrate crystals were observed in bile of ileectomized or sham-operated animals (n = 17), suggesting that all biles remained unsaturated with these solutes.

### Discussion

The most important findings in this study were that distal small bowel resection in the rat resulted in twofold increases in total biliary bilirubin outputs, whereas proximal small bowel resection, sham operation, or ileal-transection did not have this effect. Because of the use of appropriate controls, we infer that hypersecretion of bilirubin into bile strongly suggests that an induced EHC of bilirubin from the colon was the result of bile salt malabsorption. When the EHC of bile salts was closest to being intact,⁷,⁴³ at least for the ileectomized state,⁷ bilirubin secretion rates were elevated most markedly during the first 30 minutes of cannulation. This is the time period when biliary bile salt secretion rates are known to be relatively unaffected by
mechanical interruption of the EHC in the rat. In ileectomized animals, EHC of bilirubin is apparently a consequence of a surgically-induced interruption of the EHC of bile salts at the level of the distal small intestine. Hence, with continuous biliary washout, the bile salt pool within the small and large intestines became depleted, and continuous decreases in both bile salt and bilirubin secretion rates resulted (Table 1 and 2). We argue below that the dependence of bilirubin on bile salt secretion is most likely related to depletion of intraluminal bile salt concentrations in the large intestine that would impair solubilization and absorption of UCB.

A twofold elevation in biliary bilirubin levels after ileectomy (Figures 2 and 3) is in close agreement with observations of hepatic bile of the ileal-resected prairie dog by Pitt et al. Because UCB in hepatic bile was <1% of total bilirubin (Figure 1), it was not expected that the solubility product of the acid (or less likely neutral) salt of calcium and UCB would be exceeded. Accordingly, we did not observe calcium bilirubinate precipitates in any hepatic bile. Total calcium levels in bile of ileectomized rats were normal, as was noted previously in hepatic bile of the ileectomized prairie dog. However, in an animal with a gallbladder, bile composition changes markedly during concentration and storage as noted in the experiments on prairie dogs.

It is well known that, in the rat, distal small bowel resection induces hypertrophic changes in the residual intestine as early as the third postoperative day. Concomitantly, compensatory bile salt reabsorption in the proximal small intestine rapidly restores the EHC to normal. The rat, with its remarkable capacity for intestinal adaptation, allowed us to observe the reversibility of the bilirubin hypersecretory state with reestablishment of normal bile salt absorption from the small intestine over the time frame of ~1 week (Figure 3). Consistent with this experimental strategy, we observed that transient weight loss was <10% of initial body weight, diarrhea disappeared in all animals within 2-3 days, and full recovery was achieved in all animal groups by the fifth postoperative day. Because bilirubin hypersecretion was abolished completely on the 11th postsurgical day (Figure 3), it seems reasonable to assume that, because no further bile salt malabsorption took place, no intestinal absorption of bilirubin occurred after the eighth postoperative day. This is proven further with ileocolonic-transposition, in which intestinal hypertrophy was confined mainly to the proximal large intestine. In this group of animals, bilirubin secretion rates were less markedly elevated than in ileectomized animals (Figure 2),
indicating that compensatory bile salt reabsorption occurred principally in the proximal small bowel at a site where it did not contribute effectively to UCB absorption.

At first, it was puzzling that ileectomy resulted in a marked increase rather than an anticipated decrease in biliary bile salt secretion rates (Figure 3 and 4). The rat requires 2-3 days of biliary diversion to up-regulate de novo bile salt synthesis to compensate for loss; therefore contribution from new bile salt synthesis was unlikely to be the cause. Because it is well known that coprophagy provides, in part, for nutritional requirements in the healthy rat, we postulated that fecal-oral ingestion of bile salts in animals with bile salt malabsorption and diarrhea could contribute significantly to the EHC of bile salts as proven in the rabbit. This was confirmed in ileectomized animals, where the effective prevention of coprophagy reduced biliary bile salt secretion rates dramatically (Figure 4). Although coprophagy resulted in a considerable EHC of bile salts with more intestinal bile salts potentially available for reabsorption of UCB, total bilirubin secretion rates were not appreciably different between coprophagic and noncoprophagic animals (Figure 4). This suggested that tetrapyrrole degradation or formation of insoluble calcium bilirubinates in the feces or the small intestine after coprophagous ingestion prevented small intestinal absorption of UCB.

The depression of biliary bile salt secretion rates in sham-operated animals (Table 2 and Figure 2) is in accordance with similar observations in rhesus monkeys and is possibly caused by some degree of surgical shock during early postoperative periods. Because this group of animals was the only one that showed no diarrhea after surgery (see Results), it is likely that coprophagy was unable to contribute to an elevation of the low bile salt secretion rates as it did in animals with diarrhea. In ileal-transected animals, biliary bile salt secretion rates were higher than in sham-operated animals (Figure 2), most likely because of diarrhea (17% of animals) and early adaptive responses from intestinal transection.

Biliary secretion of bilirubin is determined by the hepatic flux of UCB, which can be increased by ineffective erythropoiesis, increased red cell destruction usually from intravascular hemolysis, or increased heme-destruction from nonhemoglobin sources. All of these are very unlikely causes for our observations. Increased red cell destruction is highly improbable because, in the present study, no animal developed postoperative bleeding or infection and because, in ileal-transected and -resected animals, serum analyses showed no hemolysis (see Results). Our control procedures included proximal jejunectomy and ileal resection, both of which involved
extensive surgery and operation times, but they did not increase bilirubin secretion rates into bile (Table 1 and Figure 2). This indicated that bilirubin hypersecretion was unrelated to surgery or intestinal resection per se. Furthermore, there is no a priori reason (see Berlin\textsuperscript{31}) why ineffective erythropoiesis or increased red cell destruction in the absence of bleeding and increased heme enzyme catabolism could be induced by small bowel surgery. To this end, the control animals with proximal jejunectomy and ileal-transection also rule out an induced hemolytic disorder or ineffective erythropoiesis because these operations did not result in hypersecretion of bilirubin into bile (Table 1). Furthermore, in animals with distal intestinal resections, bilirubin hypersecretion was curtailed with progressive small bowel adaptation, and was eliminated completely at 11 days after surgery (Figure 3). This renders implausible a transitory hematologic abnormality temporally related to bile salt malabsorption. Anesthesia could also be implicated as a cause of differences between the experimental groups, but this is also improbable because pentobarbital, which was used sparingly for anesthesia in our experiments, does not influence hepatic bilirubin conjugation or biliary secretion appreciably in the rat.\textsuperscript{52} Therefore, in light of these considerations, it is reasonable to conclude that the increased bilirubin secretion rate after distal small bowel resection was the product of an induced EHC of bilirubin as a consequence of bile salt malabsorption.

What then are the systemic consequences of bilirubin absorption from the large intestine? It is believed that first-pass hepatic extraction of UCB is constant, i.e., independent of load,\textsuperscript{53} so that when there is increased input from the intestines, there should be increased UCB levels in plasma. Unfortunately, the rat model is restrictive in this regard because bilirubin is virtually undetectable in normal rat plasma,\textsuperscript{54} and in both our sham and ileectomized rats, the total plasma bilirubin levels (see Results) were less than the detectable limit of \( \approx 0.1 \) mg/dL.\textsuperscript{42} Previous studies have shown that an increased UCB load reaching the parenchymal cells of the liver from any source will decrease the bilirubin diconjugate/monoconjugate ratio in bile,\textsuperscript{55} but we did not observe this. The most tenable explanation for our converse observations (Figure 2) is that it was because of augmented BMGG1 and not BDG levels that relatively more bilirubin diconjugates were secreted into bile (Figures 1 and 2). Although the reason for this was not further explored, it is highly likely that the changing fluxes of bile salts and their bacterially modified molecular species through the hepatocyte, principally from coprophagy (see below), could have affected the affinity of bilirubin uridine 5'-diphosphate-glucuronyltransferases for glucose compared with
glucuronic acid or, alternatively, the conversion of uridine 5'-diphosphate-glucose to uridine 5'-diphosphate-glucuronic acid. These beliefs are supported by the fact that, in ileectomized animals, prevention of coprophagy inhibited the fecal-oral EHC of bile salts, markedly decreased bile salt secretion rates, and concomitantly reduced biliary BMGGI levels to normal (Figure 4).

What then are the likely conditions in the cecum and large intestine that would favor bilirubin absorption in bile salt malabsorption? Lester and Schmid showed that the highly hydrophobic UCB molecule, which is insoluble when protonated, can be absorbed passively from intestinal mucosa of rats and humans in contrast to its conjugates. After absorption from the intestine, approximately 20%-30% of bilirubin is cleared from portal blood by the healthy liver in a single pass and, subsequently, efficiently conjugated and resecreted into bile. Although the UCB level in the proximal large intestine in our ileectomized animals was increased fivefold compared with ileal-transected animals (Table 3), the solubilization of UCB should have been readily facilitated and precipitation should have been prevented by the bile salt/UCB molar ratio that was approximately 500:1 (Table 3). Because UCB can be absorbed from the intestine by passive nonionic diffusion, the rate will depend on the amount solubilized and its monomeric activity. Then it seems reasonable to assume that increased colonic levels of UCB solubilized by bile salts after ileectomy (Table 3) resulted in sustained intestinal reabsorption. This concept is supported by the observation that intraduodenal infusion to rats of UCB solubilized by a high concentration of sodium dehydrocholate, a synthetic bile salt that forms dimers, produced bilirubin absorption from the gut, whereas administration of bilirubin alone did not have this effect. The discrepancy between the dramatic increase in cecal bilirubin levels (Table 3) and the at-most twofold increase in biliary bilirubin output (Figure 2) may be explained by competitive binding of UCB to undigested residue and bacteria in colonic contents, high-affinity binding to precipitated bile salt monomers, and possibly juxtamucosal unstirred water layers acting as diffusion barriers.

In health, an EHC of bilirubin is not evident so that bilirubin secretion into bile does not normally exceed bilirubin production. Physiologically, efficient homeostatic and catabolic processes in the small and large intestine prevent an EHC of bilirubin. In the upper small intestine, both the monoconjugates and diconjugates of bilirubin are too large and polar to be absorbed. In the distal small bowel and colon, bilirubin conjugates are hydrolyzed by bacterial \( \beta \)-glucuronidases, and UCB either precipitates as an insoluble calcium salt or is further
converted by bacterial catabolism into urobilinogens. Subsequently, urobilinogens may be absorbed in part from the intestine to undergo a limited EHC. Therefore, as strongly indicated by the present studies, intestinal reabsorption of bilirubin can occur only under the following conditions: (1) if bilirubin is deconjugated rapidly, (2) if bacterial degradation is slow or prevented, (3) if UCB remains in solution, and (4) if complexing with Ca\(^{2+}\) to produce insoluble salts does not occur.

As inferred from Table 3, the first of these stipulations seems to be confirmed, and the second is likely in view of the sustained bilirubin hypersecretion observed during prolonged biliary washout (Table 1). With respect to the third stipulation, although the solubility of UCB in intestinal contents depend on pH and bile salt concentration, the ratio of bile salt to UCB concentrations observed in the colon (Table 3) was sufficiently favorable to solubilize all UCB produced irrespective of pH. Finally, it is likely that the fourth point is correct because, if calcium salts of UCB had formed, they are known to be solubilized poorly by bile salts and would have precipitated intraluminally. Furthermore, the methodology used in our quantitative analysis also showed that calcium-UCB complexing was unlikely because the bile pigment was extracted easily from freeze-dried colonic contents with a cationic ion-pairing agent at a neutral pH.

It is highly unlikely that increased bile salt concentrations may modify colonic permeability to UCB, as they do in the promotion of absorption of small endobiotics and xenobiotics. For example, perfusion of the rat colon with bile salts and fatty acids is known to increase dramatically intestinal permeability to oxalate. This tiny dianion apparently diffuses down its concentration gradient through tight junctions of the colon that become sufficiently large in the presence of surfactants to admit a molecule of its size. In the case of UCB, a paracellular means of absorption would be unnecessary because of the well-known lipid solubility of UCB and its ability to “flip-flop” across biological membranes. Therefore, it seems that bile salt solubilization of diacidic UCB ranks as the most important factor favoring its passive intestinal absorption, although a limiting factor is probably the rate of UCB degradation to urobilinogens. Because elevated bilirubin secretion rates after intestinal surgery were of the same order of magnitude in ileectomized as ileocolonic-transpositioned animals (Figure 2), it seems that intestinal absorption of bilirubin can take place from any site of the proximal colon.
A final point of note is whether the small intestine could be responsible for some intestinal reabsorption of UCB, especially in the coprophagous state. Because of coprophagy, bacterial overgrowth in the small intestine of the rat is a certainty, and small intestinal deconjugation of bile salts is known to occur. Therefore, it seems reasonable to hypothesize that deconjugation of bilirubin conjugates to form UCB could also occur in the small intestine of the rat. Nevertheless, because bile salt deconjugation, precipitation, and/or reabsorption occur in this setting for the same reasons as bilirubin deconjugation, this would negate efficient solubilization of UCB by bile salts in the small intestine. Moreover, in our experiments, when coprophagy was prevented effectively, bilirubin secretion rates into bile changed insignificantly (Figure 4). This result ruled out any major contribution of the small intestine to the EHC of bilirubin during bile salt malabsorption.

Pathophysiological implications

Several biliary abnormalities in patients with Crohn’s disease speak in favour of an induced EHC of bilirubin because their gallbladder biles have low cholesterol levels and significantly increased concentrations of bilirubin conjugates and UCB. Ileal dysfunction in these patients may lead to excessive bile salt loss, and cholerrheic diarrhea may persist for long periods or indefinitely, although increased conversion of chenodeoxycholate to ursodeoxycholate may minimize catharsis. We speculate that bile salt malabsorption from any cause may lead to induced EHC of bilirubin and either hyperbilirubilia or hyperbilirubinemia, depending on the health and maturity of the liver with respect to UCB uptake, conjugation and secretion.

Caution is advisable in extrapolating the results of the present work on ileectomy to human pigment gallstone disease. The first uncertainty that arises is whether a doubling in biliary bilirubin secretion rates, as in the present work, could provide sufficient substrate for hydrolysis in the gallbladder to eventually supersaturate bile with one of the calcium salts of UCB. For example, the hemolytic nb/nb mouse is known to have a 75% prevalence of pigment gallstones within 6 months of birth; however, total bilirubin secretion rates are 10-20-fold greater than in wild strains. In humans with inherited hemolytic anemia, an order of magnitude increase in biliary bilirubin levels is also the rule.
In contrast, ileal resection in the prairie dog results in a 44% pigment gallstone prevalence in 4 weeks, but total bilirubin levels in hepatic bile are only double those of controls, which is the same magnitude of increase that we observed in our experimental rats after ileectomy (Table 1 and Figure 2). Similarly, humans with mild chronic or episodic hemolysis, e.g., malaria, prosthetic heart values, alcoholic cirrhosis, have increased prevalence rates of pigment gallstones, but elevated biliary bilirubin concentrations remain undocumented. Clearly, in animals as well as humans, residence time, bile salt concentrations, endogenous β-glucuronidase activity, ionized calcium levels, and other complex factors in gallbladder bile all play crucial roles in elevating the ion products of calcium bilirubinate salts to greater than their equilibrium solubility values.

In conclusion, we developed a rat model to monitor biliary bilirubin and bile salt secretion rates at various time intervals after a variety of intestinal surgical procedures. We show that distal but not proximal small bowel resection results in significant increases in the secretion rates of conjugated bilirubin into bile. We marshal several lines of evidence to support the argument that this is caused by enterohepatic cycling of UCB from the colon that is a consequence of an interrupted EHC of bile salts from removal of the active bile salt transport sites located in the distal small intestine. If the same pathophysiological principle holds true for humans, this would imply that enteric hyperbilirubilia may be the endogenous equivalent of enteric hyperoxaluria in bile salt malabsorption syndromes.

References


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