Portal vein bile acids in patients with severe inflammatory bowel disease

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SUMMARY The incidence of several forms of liver disease associated with inflammatory bowel disease has been putatively ascribed to a toxic effect on the liver of portal vein bile acids abnormal in type or amount. To examine this possibility, total bile acid concentrations (sulphated and non-sulphated) were measured by gas-liquid chromatography in inferior mesentric vein serum of 19 patients undergoing colectomy for severe inflammatory bowel disease (IBD). Similar determinations were obtained on a control group of eight patients requiring colectomy for other non-inflammatory conditions. Mean values for mesenteric vein serum bile acid concentrations (μM/l) were 19.6±1.8 in controls and 16.3±2.0 in IBD. The mean sulphated bile acid fraction did not exceed 10% of total, although there was considerable variability (up to 40% of total). Lithocholic acid levels (entirely sulphated in all patients) were low. Although the IBD group showed a more than two-fold increase in mean lithocholate concentration (0.54±0.15 μM/l) over controls (0.21±μM/l), this difference was not statistically significant. No significant intra-group difference was noted in the non-sulphated and sulphated fractions for cholic, chenodeoxycholic, and deoxycholic acid species, respectively. No unidentified or unusual bile acids were observed. There was no correlation between bile acid measurements and liver histology. These findings fail to support the hypothesis that liver disease often found in association with severe inflammatory bowel disease represents a form of bile acid toxicity. The invariable finding of total sulphation of the lithocholic acid fraction even in the presence of severe mucosal disease was unexpected.

At least eight forms of liver disease have been reported to occur in association with chronic ulcerative colitis (CUC), 1-3 and to a lesser degree with Crohn's disease, 4-5 especially with colonic involvement. It has been estimated, for example, that at least 50% of patients with chronic ulcerative colitis have some form of liver disease. Associated liver diseases include the following: (1) fatty liver; (2) pericholangitis of unknown aetiology; 6-8 (3) biliary and postnecrotic cirrhosis; 9-11 (4) granulomatous hepatitis; 12 (5) sclerosing cholangitis; 13 (6) chronic active hepatitis; 9 (7) carcinoma of the biliary ducts (Klatskin's syndrome); 14 and (8) amyloidosis. 15

As at least a partial explanation for the first three of the associated disease categories, it has been suggested 16 17 that a quantitative or qualitative abnormality of portal vein bile acids could result from altered intestinal bile acid absorption in presence of chronic mucosal disease. These bile acids could then exert a 'toxic' effect on the liver, as it is exposed to the bile acids contained in the portal blood draining the intestine. Another recently-published study 18 has provided useful but incomplete information on this issue. The previous study differed from the present one in that (1) the blood sampling was from the proximal portal vein region distal from that immediately draining the diseased intestine; and (2) sulphated bile acid derivatives were not measured and the measurements were, therefore, not fully quantitative. In the present study, we have

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measured all bile acids detectable by our gas chromatographic method in eight control subjects and 19 patients with severe inflammatory bowel disease.

Methods

Patients

Inferior mesenteric vein blood (usually about 10 ml) was obtained from 27 patients intra-operatively at colectomy after prior informed consent.† The region of colon for resection and its arterial and venous blood vessels were isolated and clamped. Inferior mesenteric venous blood was immediately withdrawn from the isolated vessel, and the resection procedure was then completed in the usual manner. Eight of the patients who comprised the control group (four males, four females; age range 42–80 years) had surgery performed for localised colonic lesions such as primary malignancy. The remaining 19 patients (seven males, 12 females; age range 17–59 years) required elective colectomy for severe chronic inflammatory bowel disease. Preoperative bowel preparations for all patients included administration of 3 g each of erythromycin and neomycin in divided doses during the 18 hours before surgery plus a saline bowel irrigation in the operating room. Histological study of the resected colonic tissue in these patients uniformly showed findings consistent with either mucosal ulcerative colitis or transmural colitis (Crohn’s disease). At surgery, a wedge section surgical liver biopsy was also obtained on all patients. All of the patients in the study at the time of surgery were haemodynamically stable and none showed biochemical or clinical evidence of liver disease.

Animal Studies

In four mongrel dogs weighing in excess of 15 kg colonic effluent venous blood samples were obtained at initial laparotomy. This procedure was repeated two weeks later after full recovery. An oral antibiotic regimen was administered at repeat laparotomy to simulate dosage and time of administration for human preoperative bowel preparation as employed in the present study.

Histological Studies

Colectomy tissue and surgical liver biopsies were fixed, sectioned, and stained following standard procedure for light microscopy. All slides were examined and described by a pathologist who had no knowledge of the study. In addition, single blind interpretation of liver biopsy slides from all study patients were provided by a hepatologist. Where disagreement was present these differences were resolved by consultation with a second pathologist unaware of the previous readings.

Reagents and Equipment

All solvents were either twice distilled or pesticide grade; 7-keto-deoxycholic acid, internal standard; and the reference standards, lithocholic, deoxycholic, chenodeoxycholic, ursodeoxycholic, and cholic acid were obtained from Calbiochem, La Jolla, California. Mixing was performed in a Model G24 environmental incubator shaker (New Brunswick Scientific Co. Inc., New Brunswick, NJ). Gas chromatography was performed using Model 5710A (Hewlett-Packard, Avondale, Pa) equipped with a 1·2 m (4 ft) glass column (2 mm id) packed with 1% SP-2401 on gas Chrom Q, 100/20 mesh (Supelco Inc., Supelco Park, Bellefonte, Pa).19 Column temperature was 215°C.

[Carboxyl-14C]-lithocholic acid was obtained from Amersham, Arlington Heights, Illinois, and [glycine-1-14C]-glycocholic acid was obtained from New England Nuclear, Boston, Massachusetts. Scintillation counting was performed on a LS330 liquid scintillation system (Beckman Instruments Inc., Palo Alto, California).

Measurement of Serum Bile Acids

All assays were performed in duplicate. Sample preparation before gas chromatographic analysis included in summary the following six steps: (1) deproteination, (2) delipidation, (3) separation of sulphated and non-sulphated bile acids, (4) solvolysis, (5) hydrolysis, and (6) derivatisation.

Proteins were removed by the dropwise addition of 1–3 ml serum to 10 volumes of boiling ethanol containing the internal standard, 7-ketodeoxycholic acid (10 μg/ml serum) while magnetically stirring and using a cold finger condenser to prevent evaporation. After cooling, overnight refrigeration, and centrifugation, the supernate was transferred to a tube by vacuum aspiration. The precipitate was washed twice with 10 ml boiling ethanol, both washes were added to the initial supernate, which was subsequently dried under nitrogen at 50–60°C essentially as described by Murphy et al.20

The residue was dissolved in 10 ml 50% alkaline aqueous ethanol (pH=10). Removal of netral lipids was accomplished by thrice partitioning with 20 ml petroleum ether (BP 30–60°C), shaking for 20 minutes (250 rpm), centrifuging (2000 rpm), and retaining the ethanolic phase. The ethereal phase was separated and washed with 5 ml of the alkaline aqueous ethanol and the wash combined with the ethanolic phase and dried.20

†Approval for the protocol employed was obtained from the Research Projects and Institutional Review Committee for Human Studies on 4 August 1975 and reviewed annually.
Preparation and use of Sephadex LH-20 to separate sulphated and non-sulphated bile acids was as described by Makino\textsuperscript{21} with slight modification. Non-sulphated bile acids were eluted with 100 ml chloroform-methanol (1:1, v/v) containing 0.01 M NaCl, and bile acid sulphates with 100 ml methanol containing 0.01 M NaCl; 7-ketodeoxycholic acid internal standard was added to the sulphated fraction (usually 5\(\mu\)g/ml of serum sample size). Both fractions were evaporated to dryness under nitrogen using a Rotavapor.

The sulphated bile acid fraction was redissolved in 3 ml methanol and solvolved by addition of 27 ml ethyl acetate equilibrated with 2 M aqueous \(\text{H}_2\text{SO}_4\),\textsuperscript{22} and shaking (200 rpm) at 39°C for 16 hours as described by Alme,\textsuperscript{23} then neutralised with 0.4 ml 2N NaOH and dried under nitrogen at 40°C.

The dried residues from both the sulphated and non-sulphated fractions were dissolved in 20 ml 8% NaOH in Teflon containers,\textsuperscript{24} and hydrolysed at 15 psi for three hours.\textsuperscript{23} The unconjugated bile acids were acidified to pH = 1 with 6N HCl, extracted with ethyl acetate, and neutralised with serial water washes. The ethyl acetate extract was then completely dried under nitrogen at 40°C.

The free bile acid residue was dissolved in 2 ml \(\text{CHCl}_3\text{MeOH} (1:1, \text{v/v})\), methylated in excess ethereal diazomethane for 15 minutes at room temperature, and dried under nitrogen at 37°C. The residue was dissolved in 0.2 ml methylene chloride and trifluoroacetylated with 0.2 ml trifluoroacetic anhydride at 37°C for 15 minutes. The derivatised bile acids were completely dried under nitrogen at 37°C before dissolution in 20 \(\mu\)l methylene chloride. An appropriate volume (0.1–2.0 \(\mu\)l) was then injected into the gas chromatograph.

**Calculation**

Standard calibration curves were prepared as described by Campbell.\textsuperscript{25} Each desired quantity, \(M_i\), the mass of the \(i^{\text{th}}\) bile acid in the injected serum sample, is proportional to the area, \(A_i\,\text{of the chromatographic peak that it produces. However, the peak area also depends on the particular bile acid considered. This dependence can be eliminated by measuring the ratio, \(R_i\), of peak areas produced by equal masses of the \(i^{\text{th}}\) bile acid and the internal standard. Now suppose that a known mass, \(m^*\), of the internal standard produces a peak of area, \(A^*\). Then**

\[
m_i = \frac{A_i}{R_i} \times \frac{m^*}{A^*}
\]

**Results**

A representative gas chromatographic tracing of purified standards to establish reference, \(R_i\)'s, for this study and a tracing obtained from non-sulphated and sulphated fractions of inferior mesenteric vein blood are shown in the Figure. The bile acid assay was tested for selective loss of lithocholate by adding \(^{14}\text{C}-\text{lithocholic acid to serum and removing aliquots for counting at various points during the procedure. Both deproteination and lipid extraction yielded 100% recovery of added \(^{14}\text{C}\}; 105\% of added lithocholate was recovered from the LH-20 column as the non-sulphate fraction and subsequent hydrolysis showed 91\% recovery. The final yield was 96\% of the added \(^{14}\text{C}-\text{lithocholic acid. Thus, there was no selective loss of unsulphated lithocholate using this method. Similar results were obtained using \(^{14}\text{C}-\text{glychocholate. We were unable to perform a similar direct validation for sulphated bile acids— for example, sulpholithocholate—because sulphated standards were not available. However, as we were consistently unable to detect unsulphated lithocholate in a large number of patients with detectable sulpholithocholate, we feel confident that, under our conditions, effectively complete separation of these fractions takes place on the Sephadex LH-20 column. The suitability of using the internal standard, 7-ketodeoxycholic acid for quantifying the other bile acid species measured was determined. The absolute recovery of the internal standard was 100\% of an equal mass of external standard. Thus, no further corrections were required.**

The data of Tables 1 and 2 show that the primary bile acids, cholic and Chenodeoxycholic acid, are, on average, the most abundant, comprising about two-thirds of the total. The secondary dihydroxy bile acid, deoxycholic acid, is only slightly less abundant than the primary dihydroxy bile acid, Chenodeoxycholic, and represents by far the most abundant of the secondary bile acids.

The remaining two secondary bile acids, lithocholic and Ursodeoxycholic, on average constitute at most not more than 4\% of total bile acids measured.

Differences in the degree of sulphation of the various bile acid species can be observed. The most polar of the bile acids, cholic, is least sulphated (>1\%). The dihydroxy bile acids, Chenodeoxycholic
and deoxycholic acid, show a marked variability in their pattern of sulphation. The pattern is most erratic for the chenodeoxycholic acid fraction in the experimental group where two of the patients show over 40% sulphation (JJ and AO). This results in a statistically skewed distribution in the mean sulphation measurement of chenodeoxycholic acid for this group. When the data for these two patients are excluded, the sulphation percentage in the remainder of this group is ~ 2%, comparable with that of the control group. In fact, when the values for unsulphated and sulphated bile acids of all species are compared between IBD experimental and control groups, no significant difference was found using the non-parametric Wilcoxon two-sample ranks test. The mean percentage of sulphation for the deoxycholic acid species is more consistent throughout both study groups and is notably higher (~10%) than that for the chenodeoxycholic acid fraction.

Of the two bile acids observed in relatively trace quantities, lithocholic acid is far the most abundant, being almost always detected; whereas ursodeoxycholic acid is usually undetectable. Without exception in this study—the lithocholic acid fraction was entirely sulphated. No unusual or unassignable gas chromatographic peaks potentially derived from unusual bile acids or metabolites of bile acids were observed.

In the four canine studies, colonic effluent venous serum obtained intraoperatively with and without prior antibiotic administration failed to show significant qualitative or quantitative differences in bile acid patterns. These observations showed not only the great inter-individual variability seen in the human data (Tables 1 and 2) but also considerable intra-individual variability when the paired sets data were compared. An antibiotic effect in the *acute* circumstances of their use in this study would need to be striking and this was not seen.

There were no qualitative or quantitative correlations between bile acids and liver histology from surgical biopsy. The two patients (DR and RE) in the inflammatory bowel disease (IBD) group showing the highest levels of the potentially more toxic non-

**Figure**  
(a) Gas chromatographic bile acid reference standards: (1) lithocholic, 0-10 µg; (2) deoxycholic, 0-10 µg; (3) chenodeoxycholic, 0-10 µg; (4) ursodeoxycholic, 0-10 µg; (5) cholic, 0-15 µg; (6) 7-ketodeoxycholic (internal standard), 0-20 µg.  
Support: 1%; SP-2401 on gas Chrom Q, 100/120 mesh, at 215°.  
(b) Nonsulphated bile acids in pooled inferior mesenteric vein serum (internal standard equivalent to 0-30 µg): no lithocholic is present.  
(c) Sulphated bile acids in inferior mesenteric vein serum from patient (DR) with IBD without liver disease (internal standard equivalent to 0-50 µg). Number of figures (b) and (c) correspond with those of (a). The minor peak seen in this tracing between 1 and 2 was infrequently observed and where present the mounts were too small to measure.
sulphated dihydroxy-bile acids, deoxycholic acid, and chenodeoxycholic acid, had essentially normal liver histology.

In the control group, all liver biopsy specimens were interpreted as normal and only two patients in the IBD group were judged to have histological abnormality. In one (IT), portal triaditis in the presence of extensive bile stasis was found, and the other (AH) showed scattered focal granulomas. Neither had abnormal preoperative liver function tests and both had a bile acid distribution not different from controls (Table 2).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Serum bile acid concentrations (µM/l) from inferior mesenteric vein blood in control subjects</th>
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<tbody>
<tr>
<td></td>
<td>Cholic acid</td>
</tr>
<tr>
<td></td>
<td>N</td>
</tr>
<tr>
<td>1 MB</td>
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</tr>
<tr>
<td>2 MK</td>
<td>43</td>
</tr>
<tr>
<td>3 AS</td>
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<td>7 RD</td>
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<tr>
<td>Range</td>
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<tr>
<td></td>
<td>9-4</td>
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<tr>
<td>SEM</td>
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</table>

N = nonsulphated.  
S = sulphated.  
ND = not detected.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Serum bile acid concentrations (µM/l) from inferior mesenteric vein blood in patients with inflammatory bowel disease</th>
</tr>
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<tr>
<td></td>
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</tr>
<tr>
<td></td>
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<tr>
<td>4 DR</td>
<td>7-8</td>
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<tr>
<td>5 TU</td>
<td>6-6</td>
</tr>
<tr>
<td>6 JS</td>
<td>4-3</td>
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<tr>
<td>7 SF</td>
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<td>8 JP</td>
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<tr>
<td>9 JS</td>
<td>5-6</td>
</tr>
<tr>
<td>10 CS</td>
<td>6-0</td>
</tr>
<tr>
<td>11 IT</td>
<td>2-6</td>
</tr>
<tr>
<td>12 JG</td>
<td>4-3</td>
</tr>
<tr>
<td>13 EG</td>
<td>2-3</td>
</tr>
<tr>
<td>14 UJ</td>
<td>2-6</td>
</tr>
<tr>
<td>15 AO</td>
<td>2-7</td>
</tr>
<tr>
<td>16 RE</td>
<td>13-0</td>
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<tr>
<td>17 AH</td>
<td>4-3</td>
</tr>
<tr>
<td>18 BC</td>
<td>8-5</td>
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<tr>
<td>19 WG</td>
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<td>Mean</td>
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<td></td>
<td>16-2-</td>
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<td>SEM</td>
<td>±0-9</td>
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</table>

See Table 1 for explanation of symbols.

Discussion

Most previous clinical studies on the relation of liver disease, and the concomitant presence of inflammatory bowel disease (IBD) have differed from the present work in at least three ways: (1) the data gathered were retrospective; (2) patients were selected for inclusion in the prior studies for the presence of overt clinical and biochemical or histological evidence of liver abnormality by chart review; and (3) reported patients showed an overall greater trend towards low-grade chronicity. In the
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In the present prospective study, no effort was made to include patients with clinically overt liver disease in the expectation that functional impairment would probably produce abnormalities in secondary bile acid metabolism, thus prejudicing interpretation of the bile acid measurements. In the large number of IBD patients presently reported, all had sufficiently severe and acute disease to require surgical resection. Our aim was to test the hypothesis that some would show a qualitatively or quantitatively abnormal portal vein bile acid pattern antecedent to the development of overt liver disease. In fact, we found no difference in these patients compared with controls.

Preoperative antibiotics administered only within the 18 hours before surgery are unlikely to have effected a significant alteration of portal vein bile acids in the present study. Not only did our control study in dogs in circumstances of their human use in this study fail to show an effect, but previous studies are inapplicable to present circumstances. Several previous studies, performed for different purposes have shown that striking alterations in the pattern of faecal bile acids can be induced in man with oral administration of neomycin and/or erythromycin. An important difference, however, is that in these studies, the faecal bile acid alterations were observed only after administration in high doses for several weeks. Other than the present report, no data are extant referable to possible acute effects of antibiotic administration on portal vein bile acids or on faecal bile acids.

An unexpected finding was the observation that the lithocholic acid moiety was invariably entirely sulphated. This suggests the presence of a presumably enzymatic sulphating mechanism in the colonic mucosa that is not significantly impaired even in the presence of severe inflammatory bowel disease. A clue that induction of increased polarity in comparatively hydrophobic bile acids such as lithocholate by transit through intestinal mucosa has been suggested in an earlier study. In addition, direct evidence has recently been obtained for the presence in proximal intestine of a specific bile acid sulphotransferase similar to that found in liver tissue. To our knowledge, colonic tissue has yet to be examined for a similar enzyme. Perfusion with dihydroxy bile acids has been shown to increase colonic secretion by what, in part, could also be interpreted as a cytotoxic effect. Of the two, deoxycholic is the more hydrophobic molecule as well as the more potent secretogogue. The much greater degree of sulphation of deoxycholic acid compared with chenodeoxycholic acid suggests preferential sulphation of the more hydrophobic and potentially more toxic bile acids by the colonic mucosa. The finding of invariably complete sulphation of lithocholic acid in colonic effluent inferior mesenteric vein blood can be interestingly juxtaposed against the reported estimate of about 60% sulphation of lithocholic acid found in bile obtained by duodenal drainage in two recent studies. Several alternative explanations for this could be considered: (1) small intestinal absorption of unsulphated lithocholate in significant quantity; (2) desulphation (presumably enzymatic solvolysis) during passage through the liver; (3) a degree of solvolysis occurring in the gallbladder or intestine before aspiration; or (4) an underestimate of the true degree of lithocholic acid sulphation by measurement of bile acids directly obtained from the gallbladder. Support for the last of these as the most likely explanation for this apparent discrepancy is derived from our failure to find by present methods any unsulphated lithocholic acid in five unselected gallbladder bile specimens (unpublished observations).

A comparison of the results of the present study with data from the recent literature using similar methodology is given in Table 3. The study of Ahlberg shows somewhat higher values for the three bile acid species measured but the discrepancy is not serious. We have not been able to demonstrate significant losses of internal standard through the various stages of our procedure, so this does not explain our lower values. The data of Siegel by contrast showed strikingly lower levels of all the

Table 3 Comparison of mean serum bile acid concentrations (μM/l) measured by gas chromatography from portal circuit of controls and patients with severe inflammatory bowel disease in present and two other recently published studies

<table>
<thead>
<tr>
<th>Source</th>
<th>Pts (no.)</th>
<th>Cholic acid</th>
<th>Chenodeoxycholic acid</th>
<th>Deoxycholic acid</th>
<th>Lithocholic acid</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Portal venous blood</td>
<td>10</td>
<td>6.1</td>
<td>8.4</td>
<td>7.6</td>
<td>—</td>
<td>22.1</td>
</tr>
<tr>
<td>Superior mesenteric vein</td>
<td>4</td>
<td>1.4</td>
<td>1.3</td>
<td>1.2</td>
<td>0.8</td>
<td>3.3</td>
</tr>
<tr>
<td>Inferior mesenteric vein (present data)</td>
<td>8</td>
<td>4.2</td>
<td>6.0</td>
<td>3.0</td>
<td>0.15</td>
<td>13.5</td>
</tr>
<tr>
<td>Inflammatory bowel disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superior mesenteric vein</td>
<td>5</td>
<td>2.1</td>
<td>1.7</td>
<td>1.1</td>
<td>0.5</td>
<td>5.7</td>
</tr>
<tr>
<td>Inferior mesenteric vein (present data)</td>
<td>19</td>
<td>5.7</td>
<td>4.8</td>
<td>4.3</td>
<td>0.5</td>
<td>16.3</td>
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</tbody>
</table>
bile acids compared with our present data. The earliest reported literature values for portal vein bile acid concentrations by gas-liquid chromatography are to be found in data from two control (intraoperative) observations in a study by Makino published 10 years ago. The bile acid concentrations they observed were less than half our present values. This difference is very probably a result of newer methodological improvements. The only other data on bile acids in portal vein blood relevant to the present work have been provided by the recent study of Lindblad. Control values were measured for presumably normal patients at surgery using the enzymatic fluorometric technique, though it gives only the concentration of total non-sulphated bile acids. Despite this difference in method, their mean value in 23 fasting patients of 12.9 μM/l is in fair agreement with our results.

ADDENDUM
Since the completion and acceptance of this paper, the authors have become aware of a completed study by L. Lööff and B. Wengle confirming the presence of a bile salt sulphating enzyme, glycolithocholate sulphotransferase, in different segments throughout the human small intestine and in the liver.

The authors wish to acknowledge the contributions of Dr Frank Weakley and Dr Rupert Turnbull who provided samples of inferior mesenteric vein blood from some colectomy patients in this study. They also wish to acknowledge the cooperation of Patricia A Szczepanik and Dr Peter D Klein, both of the Argonne National Laboratory who kindly provided gas chromatography-mass spectrometric analysis of some of our specimens. They are also indebted to Drs Robert H Palmer, Arnis Kuksis, Adolf Stiehl, and Stephen Barnes for helpful suggestions and useful comments regarding the procedures used and results obtained in the present study. Finally, they wish to thank Mrs Eleanor Kramer, RN, for her help in obtaining surgical specimens for this study.

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