Bile acids in the rat: studies in experimental occlusion of the bile duct

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ABSTRACT Bile acids in the plasma, urine, and small intestine of adult male rats with occluded bile ducts have been studied using a method of high specificity for their determination. After bile duct ligation cholic acid rapidly accumulates in the plasma for 8 hr, remains high for a further 8 hr, and subsequently diminishes; bile acids disappear from the small intestine. During the first 12 hr after bile duct ligation the excretion of trihydroxy acids in the urine was 10 times that of the dihydroxy acids. Subsequently the two excretion rates became equal.

Because bile acids have been implicated in the etiology of hepatic damage following bile duct ligation, studies have been made of the effect of removing (with cholestyramine) and supplementing (with cholic acid) the intestinal bile acid pool. The addition of cholestyramine to the stock diet prevented the rise in trihydroxy bile acids after bile duct ligation, but did not prevent the development of histological abnormalities in the liver. Supplementing the diet with cholic acid raised the plasma cholic acid levels but had little effect on the hepatic histological findings.

KEY WORDS bile acids * obstructive jaundice * rat * plasma * intestine * urine * etiology * hepatic necrosis * cholestyramine * cholic acid feeding

Various explanations have been given to account for the characteristic necrotic changes in the histology of the liver following bile duct obstruction (6). Among these are suggestions that bile under pressure (7) may be the causative agent; there have also been suggestions that bile salts (8) are the hepatotoxic agents.

The resin cholestyramine binds bile salts at the pH of the small intestine (9). We have compared the plasma bile acid concentrations and histological changes in rats on the stock diet with those receiving a cholestyramine supplement prior to bile duct ligation.

In addition bile acids were fed in the stock diet at concentrations of 0.10–2% to observe the effect of dietary bile acids on the plasma bile acid concentration. A study was made of the histological changes in the livers of these animals which had increased plasma bile acid concentrations, but patent common bile ducts.

METHODS

The rats in these experiments were adult male Wistar strain animals bred in the laboratory colony and weighing approximately 200 g. The stock diet consisted of 70% whole wheat flour, 25% milk powder, and 5% brewer's yeast. In the appropriate experiments cholestyramine or cholic acid was added to this stock diet. Cholestyramine is a quaternary ammonium anion exchange resin with the basic groups attached to a styrene-divinylbenzene copolymer skeleton which at the pH of the small intestine binds bile salts (9).

The animals were anesthetized with ether and the common bile duct was exposed, doubly ligated, and divided under clean but not sterile conditions. Sham experiments were carried out under the same conditions except that the patency and continuity of the common bile duct was not impaired.
The animals were killed at fixed intervals postoperatively and at least four animals were used at each interval varying from 30 min to 96 hr.

Blood samples were obtained by cardiac puncture using syringes containing heparin. The plasma was separated and frozen until used. The entire small intestine from the pylorus to the ileocecal valve was removed and quickly cut into small pieces in alcohol.

In the experiments involving urine collection contamination of the urine by fecal bile acids was avoided. Individual rats were kept in appropriately sized Büchner funnels on fine mesh wire which prevented the feces from dropping into the collection vessel.

Histological studies were made of the medial and lateral lobes of the liver of each animal. The tissues were fixed in 10% buffered formalin and sections were stained by hematoxylin and eosin and where necessary by Scharlach R.

**Cholestyramine Feeding Experiments**

Adult male rats (approximately 200 g) were placed on the stock diet supplemented with 2% cholestyramine, so that each rat ate about 300 mg of the resin per day. The rats were kept on this diet until they developed steatorrhea, which was indicative that sufficient bile salts were being removed to impair the absorption of lipids. At this stage the common bile duct was ligated as described above.

**Cholic Acid Feeding Experiments**

Adult male rats were fed diets containing cholic acid at concentrations ranging from 0.10 to 2% per day for 10 days. The animals were anesthetized with ether; blood was removed by cardiac puncture in syringes containing heparin and the plasma was separated. The livers were removed for histological examination as described previously.

**Bile Acid Estimations**

The estimations were made on plasma, small intestine, or urine, using the method of Reid and Boyd (5). Figure 1 shows the procedure for the extraction of bile acids from plasma; some modifications are made for the estimation in other tissues.

The chloroform–ethanol extracts obtained were taken to dryness, and the residue was dissolved in a fixed volume (usually 2.0 ml) of 70% acetic acid. An aliquot of the acetic acid solution was subjected to partition chromatography in order to separate mono-, di-, and trihydroxyalted bile acids (10).

The columns used for plasma bile acids consisted of 6 g of acid-washed Celite which had been made into a slurry with 4 ml of 70% acetic acid previously equilibrated with petroleum ether (bp 60–80°). The elutions with made with petroleum ether (bp 60–80°) and benzene in graded mixtures each previously equilibrated with 70% acetic acid. Each elution volume was 25 ml except for the final elution with pure benzene, which was 50 ml. The monohydroxylated bile acids were eluted with petroleum ether, dihydroxy acids with benzene–petroleum ether 40:60 (ν/ν), and the trihydroxy acids with the first portion of pure benzene. The column does not effectively fractionate the different tri- and dihydroxy acids. A typical chromatogram is shown in Fig. 2.

Thin-layer chromatography (TLC) was carried out on the individual fractions from the column using the systems proposed by Eneroth (12). The plates were sprayed with concentrated sulfuric acid and heated. In high concentrations cholic acid, 3α,6β,7β−, 3α,6α,7α−, and 3α,6β,7α−trihydroxy acids all gave yellow spots. The 6-hydroxylated acids gradually became purple with heating, a color change which seemed to be accentuated when very small amounts were present.

The tubes containing the column fractions were heated for 18 hr at 137°, as traces of acetic acid from the column seriously affected the subsequent color reaction—a modified Pettenkofer reaction for dihydroxy and trihydroxy bile acids. Five milliliters of 70% sulfuric acid was added to the dried bile acids from the eluates, followed 5 min later by 1.0 ml of 0.25% aqueous furfural solution. A pink color appears which takes 60 min to develop to maximum intensity (A = 0.6 for 45 μg of cholic acid), after which the color fades slowly. Readings were made at the absorption maximum of 510 mμ. A graph of optical density versus cholic acid concentration was linear in the range 5–100 μg.

It was necessary to establish the specificity of the color reaction used for the various bile acids liable to be found in the body fluids of the rat. The chromogenicity and chromatographic elution characteristics of a number of bile acids are shown in Table 1. All the compounds shown in the table ran as single spots on TLC. It will be seen that many bile acids are Pettenkofer-positive, but notable exceptions are lithocholic acid and the muricholic acids.

Thin-layer chromatography of the eluates showed that the principal bile acids in rat plasma and small intestinal content are chenodeoxycholic and cholic acids, which were therefore used to construct the calibration curves for dihydroxy and trihydroxy acids respectively. The nonspecific background color was low, so that in the plasma bile acid estimations the average background for the entire series could be subtracted from the peak area without introducing an appreciable error. Table 2 shows that standard amounts of sodium taurocholate (and of sodium chenodeoxycholate, not shown) gave recoveries of 80–90% in the 10–200 μg range.
**Bile Acids of the Small Intestine**

The small intestine, including the wall and contents, was either homogenized in a blender (Measuring & Scientific Eqpt., London) or cut into a fine mince with scissors before extraction with ethanol. Three cold extractions were performed and finally the tissue was refluxed with boiling ethanol for 2 hr. The ethanol extracts were filtered and combined. The subsequent procedures were exactly as described for the plasma bile acid determinations.

**Urine Samples**

Urine samples were collected directly into 5 N sodium hydroxide (which was then adjusted to 2.5 N), and hydrolyzed as described for the plasma bile acids.

**Other Estimations**

Total and free cholesterol estimations were performed by the method of Sperry and Webb (13). The plasma glutamic–pyruvic transaminase activity was determined by Reitman and Frankel’s method (14).

**TABLE 1. RELATIVE CHROMOGENICITIES OF VARIOUS BILE ACID DERIVATIVES IN THE MODIFIED PETTENKOFER REACTION**

<table>
<thead>
<tr>
<th>Cholanoic Acid</th>
<th>Eluent Benzene–Petroleum Ether</th>
<th>Pettenskof Chromogenicity per 10 µg of Bile Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>3α,7α,12α</td>
<td>80:20</td>
<td>0.137</td>
</tr>
<tr>
<td>3α,7,12α,12α</td>
<td>80:20</td>
<td>0.05</td>
</tr>
<tr>
<td>3α,12α delta 8–14</td>
<td>40:60</td>
<td>0.05</td>
</tr>
<tr>
<td>3α,7α</td>
<td>60:40</td>
<td>0.06</td>
</tr>
<tr>
<td>3α,12α</td>
<td>60:40</td>
<td>0.04</td>
</tr>
<tr>
<td>3α,7β</td>
<td>60:40</td>
<td>0.008</td>
</tr>
<tr>
<td>3α,7α,12-oxo</td>
<td>80:20</td>
<td>0.003</td>
</tr>
<tr>
<td>3α,12α,7-oxo</td>
<td>80:20</td>
<td>0.0007</td>
</tr>
<tr>
<td>3α,7-oxo</td>
<td>40:60</td>
<td>0.001</td>
</tr>
<tr>
<td>3α,12-oxo</td>
<td>40:60</td>
<td>0.001</td>
</tr>
<tr>
<td>3α</td>
<td>0:100</td>
<td>0.0008</td>
</tr>
</tbody>
</table>
RESULTS

After the bile flow from the liver to the duodenum had been arrested, the plasma bile acid concentrations abruptly changed. Half an hour after ligation the plasma trihydroxy (cholic) acid concentration was 20 times the initial value and the concentration continued to rise to a maximum at 8 hr of approximately 12 mg/100 ml (Fig. 3). This level was maintained for a further 8 hr, after which there was a gradual fall during the next 56 hr. The dihydroxy acids increased about 8-fold after 30 min, then remained at this same level for the remainder of the experiment.

The individual eluates from chromatographic separations of the bile acids in these plasmas were examined...
Changes in the amount of bile acids in the entire small intestine (wall + contents) after ligation of the common bile duct. ○—○ trihydroxy and ⋄—⋄ dihydroxy bile acids.

After bile duct ligation bile acids rapidly disappeared from the small intestine (Fig. 4). There was a 92% fall in the trihydroxy bile acids in the small intestine during the 8 hr following the operation.

During the first 12 hr, 5 mg of trihydroxy acid and 0.5 mg of dihydroxy acid appeared in the urine (Fig. 5). During the subsequent 12 hr the urinary dihydroxy bile acids increased 5-fold, whereas the outflow of trihydroxy acids was halved. Over the next 48 hr the dihydroxy acids declined to half; and the trihydroxy acids declined to about one-fifth of that found in the first 12 hr. In the period of 72 hr after bile duct ligation over 10 mg of cholic acid per rat was excreted into the urine. This figure is similar to that found by Mahowald et al. (11), who also found considerable amounts of other (Pettenkofer-negative) bile acids to be present in the urine of surgically jaundiced animals.

The urine collected from rats during the first 12 hr after bile duct ligation contained lithocholic acid and also material chromatographically identified as cholesterol.

The dihydroxy peak was composed of a mixture of chenodeoxycholic acid, deoxycholic acid, a slightly less polar substance (possibly 3α,12α-dihydroxy-5α-cholanoic acid), and a compound similar to 3α,7β-dihydroxy-5β-cholanoic acid. The trihydroxy peak on further examination by TLC revealed a purple staining spot which was also present in the 80% benzene eluate. This material was Pettenkofer-negative and may be 3α,6β,7β-trihydroxy-5β-cholanoic acid. The trihydroxy eluate when subjected to analysis on TLC ran as a single spot and the UV absorption spectrum in sulfuric acid was almost identical with that obtained with cholic acid.
The bile acids from the urine of bile duct-ligated animals collected in the period 12–24 hr after the operation were examined by TLC. There were now several components present in the 100% petroleum ether eluate which were not present in the earlier urine collections. However, the principal bile acid was still lithocholic acid. The bile acids found in the dihydroxy and trihydroxy bile acid peaks were essentially the same as described for the 0–12 hr period. The 100% benzene eluate contained a substance which on TLC behaved like 3α,6β,7α-trihydroxy-5β-cholanoic acid.

For the remaining 48 hr of the experiment the only change observed in the urinary bile acids was that chenodeoxycholic acid could not be detected in the dihydroxy peak. There was, however, material present in the dihydroxy eluate which behaved on TLC the same as 3α-hydroxy-12-keto-5β-cholanoic acid. Since the bile salts are rapidly reabsorbed from the small intestine it is not surprising that the change in the plasma bile acids initially reflects the pattern of bile salts found in the small intestine. The peak plasma concentration of cholic acid after bile duct ligation occurs at about 6–8 hr postoperatively and hence the bile acids in the urine during the first 12 hr postoperatively are mainly cholic acid and chenodeoxycholic acid. The bile acids which occur normally in the cecum are reabsorbed much more slowly and these products of microbiological attack appear in the urine only after 12 hr.

The gradual increase in the plasma cholesterol, largely as free cholesterol (Fig. 6) is in accordance with previous observations (15). This increase in plasma cholesterol concentration is slow to develop: it was almost 12 hr before the cholesterol concentration in the plasma was significantly elevated.

On the other hand, within 2 hr after duct ligation the plasma glutamic–pyruvic transaminase (P.G.P.T.) activity was approximately 100 units and subsequently rose to even higher values (Fig. 7).

**Sham Ligation**

During these experiments the sham-ligated animals showed small but significant variations in the trihydroxy bile acid content of the small intestine. Less frequently, the plasma bile acid concentration was slightly raised, with no evidence of accidental obstruction during the sham operation procedure.

**Cholestyramine-Fed Rats**

Evidence that the elevation of plasma bile acids is a direct consequence of the diversion of the enterohepatic circulation into the systemic circulation is given by the experiment with cholestyramine-fed rats (Fig. 8). The rats had been fed 2% cholestyramine for 12 days prior to the occlusion of the bile duct. Even 48 hr after bile duct ligation there were only small quantities of trihydroxy bile acids in the plasma, but the dihydroxy

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**Fig. 5.** Urinary excretion of bile acids following ligation of the common bile duct.
acids followed their usual pattern. It seems possible that the cecal flora may be able to remove bile salts from the resin and consequently some of the cecohepatic circulation is maintained. Normally the hydroxylation of deoxycholic acid at the 7-position is sufficient to give quantitative reversion of this acid to cholic acid (16); however, the increase in the plasma dihydroxy bile acids suggests that under these particular experimental

FIG. 6. Changes in the plasma cholesterol after ligation of common bile duct. ○—○ total; ●—● free.

FIG. 7. Changes in the plasma glutamic-pyruvate transaminase activity after ligation of the common bile duct. ○—○ cholestyramine diet; ●—● stock diet.

FIG. 8. Changes in plasma bile acids following bile duct ligation in animals fed the stock diet and in those fed cholestyramine in the stock diet.
Within 2 hr after bile duct ligation the plasma glutamic-pyruvic transaminase activity rose to about 20 times the value found in the normal rat plasma (Fig. 7). The number of rats in the series (six rats per group) was too small to bear adequate statistical analysis but 2 hr postoperatively enzymatic activities were recorded which were more than twice the values found in bile duct-ligated rats fed the stock diet. Therefore, in the absence of measurable trihydroxy bile acids from the plasma, there is considerable release of transaminase activity into the plasma. This finding is difficult to explain if cholic acid is to be implicated as an important source of liver damage in the duct-obstructed animal.

Feeding Experiment
If cholic acid is fed in the diet to rats at concentrations of 0.10–2.0%, after 10 days there are considerable amounts of trihydroxy acids in the plasma (Fig. 9). There is a fairly linear relationship between the cholic acid in the diet and the observed plasma cholic acid concentrations. Supplementing the diet with 2% of cholic acid produces an increase of the estimated pool size to 30 times the normal value. Under these circumstances the plasma level is around 5 mg/100 ml, which is about the value found for rats 30 min after common bile duct ligation.

Histological Observations
The abnormalities which appeared in the liver after ligation of the common bile duct were, in brief, edema of the portal tracts, hyperplasia of the bile ducts in the portal triads, and patchy degeneration of liver parenchyma followed by regenerative activity in liver cells. The changes were graded for extent and severity in individual sections. Some variation was found not only between the livers of animals studied at the same interval after duct ligation, but even between different lobes of the same liver. Nevertheless the trend was unmistakable and the sequence of events, which is similar to that observed by previous workers (17), may be summarized as follows. A section of a normal liver is shown for comparison (Fig. 10).

Edema of the Portal Tracts
Edema of the portal tracts was obvious 30 min after duct ligation, reached a maximum at 16–24 hr, and persisted throughout the experiment (Fig. 11).

Changes in the Bile Duct of the Portal Triads
The ducts were slightly swollen at 1 hr, and swelling and irregularity were commonly found at 4 hr. Hydrops of the nuclei were observed at 8–12 hr. Mitotic activity and marked hyperplasia were present at 24 hr. The mitotic activity declined after 72 hr but hyperplasia persisted throughout.

Hepatic Necrosis
Hepatic necrosis most often took the form of irregularly distributed areas of lytic necrosis involving up to two to three lobules. They first appeared at 4 hr, were moderately numerous at 12–24 hr, and thereafter became less frequent. A few animals showed larger areas of coagulative necrosis which were most marked in subcapsular zones, but the finding was inconstant (Figs. 12, 13).
Fig. 10. Section of normal liver of rat.
Fig. 11. Edema of the portal tract, 2 hr after ligation of the common bile duct.

Fig. 12. Focal necrosis of liver parenchyma, edema of portal tract, and bile duct hyperplasia, 24 hr after ligation of the common bile duct.

Fig. 13. Portal tract showing proliferation of the bile duct, 48 hr after ligation of common bile duct.
Figs. 14–17. Hematoxylin and eosin.

Fig. 14. Mitotic figures (magnified) from central left-hand edge of Fig. 17. × 450.

Fig. 15. Liver section from rat fed cholestyramine, 2 hr after common bile duct ligation. × 130.

Fig. 16. Liver section from rat fed cholestyramine, 24 hr after common bile duct ligation. × 130.

Fig. 17. Liver section from rat fed cholestyramine, 48 hr after common bile duct ligation. × 130.
Mitotic Activity in Liver Cells

Mitotic activity in liver cells appeared (Fig. 14) at 48 hr and was then usually moderate (three or four mitotic figures in a high-power field). It declined slightly at 72 and 96 hr.

Effect of Cholestyramine

The histological changes present 2–48 hr after bile duct ligation were not modified by administration of cholestyramine (Figs. 15–17). The livers of rats fed cholestyramine (with patent bile ducts) were normal (Fig. 19).

Effect of Dietary Supplements of Cholic Acid

In some of the livers there was slight fatty change, and in some there were other slight abnormalities, which, however, were inconstant and of doubtful significance. There were no changes resembling those resulting from bile duct ligation (Fig. 18).

DISCUSSION

Using the method of Reid and Boyd (5) bile salts have been extracted and separated by a partition chromatography, and the dihydroxy and trihydroxy bile acids have been estimated by the use of a modified Pettenkofer reaction. Studies have been made of the changes in plasma bile acids produced by experimental occlusion of the common bile duct in the male adult rat.

After ligation of the bile duct the bile acids reaching the liver from the intestine are diverted to the extracellular fluid and accumulate rapidly in the plasma. The rate of this accumulation is perhaps accentuated in the rat because it has no gallbladder; the normal capacity of its biliary system is sufficient to hold, without distention, only the volume of bile formed in about 2 min (6).

The increase in the plasma bile acids and the appearance of bile acids in the urine are due to the rapid reabsorption of bile acids from the small intestine. During the first 8 hr after the enterohpatic circulation has been interrupted, the bile acid content of the small intestine is depleted and during the same period the plasma bile acids reach a maximum concentration. The continued increase in the cholic acid concentration for a further 8 hr is due in part to the 7α-hydroxylation of reabsorbed deoxycholic acid from the cecum. The total urinary bile acid excretion during the first 24 hr is about 400 μg/hr, which is twice that for the succeeding 24 hr and four times that for the period 48–72 hr postoperatively. The relatively small increase in the plasma dihydroxy bile acids during the first 8 hr is reflected by the appearance of only small amounts of dihydroxy bile acids in the urine in this period. The ratio of trihydroxy to dihydroxy bile acids in the small intestine is

1 Boyd, G. S., and M. A. Eastwood, unpublished results.
3:1 and yet only a small proportion of the dihydroxy acids was excreted. Most of the reabsorbed chenodeoxycholic acid will be 6β-hydroxylated to become 3α,6β,7α-trihydroxy-5β-cholanoic acid which is not determined in the present method. In the succeeding 8 hr some deoxycholic acid (of cecal origin) appeared in the urine, which suggests a failure of the hepatic 7α-hydroxylation mechanism.

When the bile acid sequestering agent cholestyramine was fed to animals steatorrhea occurred. This is taken as evidence that the bile salts in the small intestine were bound to the resin and hence were no longer available for a role in lipid absorption. After bile duct occlusion in these animals there was no rise in the plasma cholic acid, presumably because the cholic acid conjugates in the small intestine were firmly bound to the resin. The possibility exists (in bile duct-ligated animals) of transmural leakage of bile salts from the extracellular fluid to the intestinal lumen where they may be adsorbed on to cholestyramine.

We compared some of the other changes in bile duct-ligated animals fed either the stock diet or the cholestyramine-supplemented diet. Despite the reduced plasma bile acid level found in rats fed cholestyramine (before bile duct occlusion) the histological changes produced in the livers of these animals could not be distinguished from those in similarly operated animals fed on a stock diet. The plasma transaminase enzyme activities of the cholestyramine-fed rats were about twice as high as the values found in animals not fed the resin but subjected to bile duct ligation.

Holsti (18) has indicated that rabbits fed chenodeoxycholic acid or lithocholic acid as small supplements to their diet developed cirrhosis.

In our ligation experiments the dihydroxy acids were found at concentrations of 2–3 mg/100 ml within 1 hr of the ligation whereas in the feeding experiments the concentration was less than 1 mg/100 ml. However, it is significant that in the rabbit (the species used by Holsti), there is no 7α-hydroxylation mechanism for cholanoic acid and furthermore the rabbit normally tolerates a plasma concentration of deoxycholic acid in excess of 2.0 mg/100 ml (19). The cirrhotogenic activity of chenodeoxycholic acid may be mediated through its conversion to lithocholic acid (20) by intestinal bacteria since there is no hepatic rehydroxylation to the original chenodeoxycholic acid.

It appears from our work that cholic acid per se does not cause hepatic lesions in bile duct-ligated rats.

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