Ursodeoxycholic acid, chenodeoxycholic acid, and 7-ketolithocholic acid are primary bile acids of the guinea pig

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Abstract Guinea pig gallbladder bile contains chenodeoxycholic acid (62 ± 5%), ursodeoxycholic acid (8 ± 5%), and 7-ketolithocholic acid (30 ± 5%). All three bile acids became labeled to the same specific activity within 30 min after [³H]cholesterol was injected into bile fistula guinea pigs. When a mixture of [³H]ursodeoxycholic acid and [¹⁴C]chenodeoxycholic acid was infused into another bile fistula guinea pig, little ³H could be detected in either chenodeoxycholic acid or 7-ketolithocholic acid. But, ¹⁴C was efficiently incorporated into ursodeoxycholic and 7-ketolithocholic acids. Monohydroxylated bile acids make up 51% and ursodeoxycholic acid 38% of fecal bile acids. After 3 weeks of antibiotic therapy, lithocholic acid was reduced to 6% of the total, but ursodeoxycholic acid (5-11%) and 7-ketolithocholic (15-21%) acid persisted in bile. Lathosterol constituted 19% of skin sterols and was detected in the feces of an antibiotic-fed animal. After one bile fistula guinea pig suffered a partial biliary obstruction, ursodeoxycholic and 7-ketolithocholic acids increased to 46% and 22% of total bile acids, respectively. ▪▪ These results demonstrate that chenodeoxycholic acid, ursodeoxycholic acid, and 7-ketolithocholic acid can all be made in the liver of the guinea pig. —Tint, G. S., G. Xu, A. K. Batta, S. Shefer, W. Niemann, and G. Salen. Ursodeoxycholic acid, chenodeoxycholic acid, and 7-ketolithocholic acid are the primary bile acids of the guinea pig. *J. Lipid Res.* 1990. **31:** 1301–1306.

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The bile of both the guinea pig and the nutria (*Myocastor coypus*) contains the three dioxygenated bile acids chenodeoxycholic acid (chenodiol), ursodeoxycholic acid (ursodiol), and 7-ketolithocholic acid, but only trace quantities of the trihydroxylated bile acid cholic acid (1-8). In the guinea pig chenodeoxycholic acid is the most abundant bile acid, followed by 7-ketolithocholic acid and ursodeoxycholic acid, in that order (1-6). Nutria, in contrast, have bile in which the concentrations of ursodiol and 7-ketolithocholic acid are more nearly equal and chenodiol is usually the least plentiful of the three (7, 8). Danielsson et al. (2, 3) have postulated that chenodeoxycholic acid is the primary guinea pig bile acid and that during its passage through the intestine a portion is oxidized to 7-ketolithocholic acid. It is assumed that the 7-ketolithocholic acid made by the bacteria is then reabsorbed and transformed to ursodiol in the liver of the animal.

It is known that 7-ketolithocholic acid can be reduced to both chenodiol and ursodiol in the liver of several mammalian species (2, 3, 7, 9-14) as well as by several species of the bacterium *Clostridia* (15-17) and that 7-ketolithocholic acid is the major intermediate in the conversion of chenodeoxycholic acid to ursodeoxycholic acid in the human colon (18). However, other evidence suggests that the above model of bile acid formation in the guinea pig may be incomplete. In general, the intestinal flora found in mammals are far more likely to cleave the C-7 hydroxyl group than to carry out the corresponding C-7 dehydrogenation (17, 19). The intestinal bacteria of the guinea pig are probably not much different than those in other species because, as we shall demonstrate below, the major guinea pig fecal bile acids are C-3-monohydroxylated bile acids. It has also been reported that the administration of cholestyramine, which binds bile acids in the gut and prevents their return to the liver, either does not alter the relative concentration significantly (4) or reduces the relative concentration of biliary ursodiol to a much greater extent than it reduces biliary 7-ketolitho-

cholic acid (6). Therefore, as we have demonstrated previously that chenodiol, ursodiol, and 7-ketolithocholic acid can all be synthesized directly from cholesterol in the liver of the nutria (7), there is precedent for suggesting that the same might be true of the guinea pig.

We describe here a series of experiments that demonstrate that the guinea pig is fully capable of synthesizing ursodeoxycholic acid and 7-ketolithocholic acid hepatically. In other words, both ursodeoxycholic acid and 7-ketolithocholic acid are primary bile acids where the term "primary bile acid" means a bile acid that is made wholly within the liver, but without the intervention of bacteria. This definition would encompass bile acids made from bile alcohol as well as bile acid precursors.

MATERIALS AND METHODS

Radioactive compounds

[24-^{14}C]Chenodeoxycholic acid (52 mCi/mmol), [11,12-^{3}H]ursodeoxycholic acid (37 Ci/mmol), and [1,2-^{3}H]cholesterol (50 Ci/mmol) were purchased from NEN Research Products, Boston, MA.

Thin-layer chromatography (TLC)

Bile acid methyl esters were chromatographed on silica gel G plates (Type O, The Foxboro Company, North Haven, CT) developed in chloroform–acetone–methanol 70:20:5 (vol/vol/vol).

Gas–liquid chromatography (GLC)

The trimethylsilyl ether derivatives of bile acid methyl esters and neutral sterols were injected on 25 m × 0.25 mm ID, 0.25 μm film thickness, CP-Sil-5 CB fused silica columns (Chrompack Inc, Bridgewater, NJ). The columns were operated at a helium flow of 0.9 ml/min and a split of 20:1. The initial temperature was set to 100°C for 2 min and then raised to 280°C at a rate of 35°C/ min (20). In this particular system lithocholic acid and its 3β-OH epimer, isolithocholic acid, do not resolve but cholic acid, chenodiol, ursodiol, and 7-ketolithocholic acid were baseline-separated.

Mass spectrometry

Capillary GLC mass spectrometry was carried out on a Hewlett-Packard model 5988 mass spectrometer operating with an electron energy of 70 eV and using GLC conditions similar to those described above.

Specific activity measurements

After purification of bile acids by TLC, the radioactivities in chenodiol, ursodiol, and 7-ketolithocholic acid were determined by liquid-scintillation counting while the mass was measured by GLC.

Analysis of bile acids and neutral sterols in bile and feces

Samples were processed as described previously (7). One to 2 ml of bile were deproteinized by passage through a C$_{18}$ Sep-Pak cartridge (Waters Associates, Milford, MA); the bile acids were then deconjugated enzymatically and again deproteinized.

Feces as well as a sample of guinea pig chow were lyophylized and ca. 1 mg was extracted overnight with 1% NH$_4$OH in ethanol in a Soxhlet apparatus. The ethanol was evaporated, aqueous NaOH (pH 12) was added, and the neutral sterols were extracted with hexane. The water layer from the feces was acidified to pH 1–2 with concentrated HCl and the bile acids were extracted with ethyl ether. To determine the percentage of conjugated bile acids in the feces of antibiotic-fed animals, the aqueous layer was divided in half. One portion was acidified and the bile acids were extracted as above. The second fraction was made alkaline with NaOH to yield a 3 N solution and then heated at 125°C for 3 h to hydrolyze any conjugates. The sample was then acidified and the free bile acids were extracted (7). Bile acid methyl esters were formed by overnight treatment with 3 N HCl methanol.

Skin sterols were assayed by wiping the fur of an animal several times with glass wool moistened with acetone and then hydrolyzing the residue by refluxing the glass wool at 70°C for 1 h in 1 N NaOH.

Bile acid and neutral sterol concentrations were measured by GLC using methyl ursocholate (methyl 3α,7β,12α-trihydroxy-5β-cholanoate) and 5α-cholestane as internal standards.

Experimental design and operative procedure

Guinea pigs (male retired breeders, 890–1250 g were anesthetized with an intramuscular injection of ketamine, 30–40 mg/kg body weight, and a 0.86 mm ID × 1.27 mm OD polyethylene catheter was placed in the common bile duct. A 0.58 mm ID × 0.95 mm OD catheter was placed in the jugular vein and lactated Ringer's solution was infused at a rate of 10–15 ml/h. All tubing was passed through a dorsal incision so that the animals did not have to be restrained during the experiments. Radiolabeled compounds were dissolved in 0.2 ml ethanol, mixed with 0.5 ml saline, and infused rapidly into the jugular catheter.

To determine the time required to completely drain the preexisting bile acid pool, 2.5 μCi of [^{14}C]chenodiol was injected into two animals immediately after creating a bile fistula. Consecutive 4-h pools of bile were collected and the radioactivity, as well as the bile acid composition and concentration, in each portion was measured.

Because it has been postulated that 7-ketolithocholic acid is a product of intestinal bacteria (2), 100 mg chloro-

mycetin and 25 mg metronidazole were administered two and five times a day, respectively, for 3 weeks to two guinea pigs via a 0.58 mm ID polyethylene tube placed in the stomach surgically. At the end of this period a bile fistula was created in these animals. Gallbladder bile and feces were obtained before and after treatment and the bile acid composition was determined.

The ability of the liver of the guinea pig to convert cholesterol to chenodiol, ursodiol, and 7-ketolithocholic acid was evaluated by injecting 5.9 μCi of [^{3}H]cholesterol into the jugular vein of three animals (two control and one antibiotic-fed) immediately after cannulating the bile duct. We also investigated the possibility that chenodiol might be transformed to ursodiol and 7-ketolithocholic acid and that ursodiol could be converted to chenodiol and 7-ketolithocholic acid hepatically by injecting 4.5 μCi [^{14}C]chenodiol plus 8.0 μCi [^{3}H]ursodiol into an animal immediately after preparing the bile fistula. In both experiments, bile was collected hourly and the specific activities of each of the three bile acids were measured.

Bile from the guinea pig which developed a partial biliary obstruction was cultured in the hospital clinical laboratory using standard techniques for the growth of both aerobic and anaerobic organisms.

All animal experiments were approved by the Animal Research Committees of the VA Medical Center, East Orange, NJ and the New Jersey Medical School, Newark, NJ.

RESULTS

Chenodiol, ursodiol, and 7-ketolithocholic acid constituted 62 $\pm$ 5%, 8 $\pm$ 5%, and 30 $\pm$ 5% (mean $\pm$ SD), respectively, of total gallbladder bile acids from eight animals. Twelve to 16 h after complete biliary diversion was achieved these percentages changed to 73 $\pm$ 7%, 5 $\pm$ 3%, and 21 $\pm$ 5%, respectively. The relative concentrations of ursodiol and 7-ketolithocholic acid continued to decline slowly with time and finally disappeared in two animals after 96 h. However, 7-ketolithocholic acid persisted in the bile (7-ketolithocholic acid: 9%, chenodiol: 93%) in a third guinea pig even after 120 h of complete biliary diversion. ^{14}C became undetectable in the bile from two animals within 48 and 72 h, respectively, after they were injected with 2.5 μCi [^{14}C]chenodiol and a mean of 100% of infused label was collected. Thus, the endogenous bile acid pool in the guinea pig is entirely depleted 2–3 days after a bile fistula is created. Biliary secretion began to diminish in one of the animals at the end of 72 h of total biliary diversion, after all label had disappeared (**Fig. 1**). Bile flow fell from a mean of 5.6 $\pm$ 1.4 ml/h (hours 1–72) to 1.0 ml/h after 7 days. At the same time, bile acid secretion declined from 1.04 $\pm$ 0.28 μmol/h (hours 8–72) to 0.18 μmol/h (day 7) and the
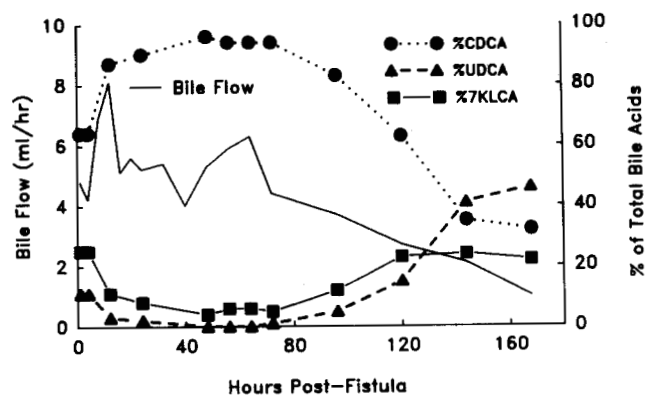

Fig. 1. Bile flow (left axis) and relative concentrations of chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), and 7-ketolithocholic acid (7KLCA), as percent of total bile acids, (right axis) as a function of time in the bile of a guinea pig with total biliary diversion that developed a partial biliary obstruction after 72 h.

bile became increasingly opaque, viscous, and brownish colored. With the other bile fistula guinea pigs, the relative concentrations of chenodiol (CDCA), ursodiol (UDCA), and 7-ketolithocholic acid (7KLCA) changed from 64%, 11%, and 25% to 95%, 0%, and 5%, respectively, after about 72 h. However, as the partial biliary obstruction became manifest and bile flow began to diminish, the percentages of ursodiol and 7-ketolithocholic acid began to increase steadily and became equal to 46% and 22% of total bile acids, respectively, after 7 days (Fig. 1). This latter composition resembles that of the nutria (7, 8), not the guinea pig. At this time chenodiol, ursodiol, and 7-ketolithocholic acid were being secreted at a rate of 0.6, 0.8, and 0.4 mg/day, respectively. Bile obtained on day 7 was cultured and found to be sterile.

Before the animals were treated with antibiotics, the major bile acids, in the feces were the C-3 monohydroxylated lithocholic acid and/or isolithocholic acid (51%). Ursodiol (38%) was also present together with small quantities of chenodiol (5%) and 7-ketolithocholic acid (6%). Biliary bile acids in the two animals consisted of 52% and 58% chenodiol, 17% and 5% ursodiol, and 31% and 38% 7-ketolithocholic acid, respectively. The predominant fecal neutral sterols were found to be the bacterially 5β-saturated products of cholesterol, sitosterol, and campesterol. Cholesterol and sitosterol, themselves, accounted for only about 20% of the total. After 3 weeks of antibiotic therapy the relative concentration of the lithocholic acids fell to 6% of total fecal bile acids while chenodiol, ursodiol, and 7-ketolithocholic acid increased to 15%, 68%, and 11%, respectively. Before treatment none of the fecal bile acids were amidated but, at the end of the treatment period, 70% of the bile acids was found to be conjugated. After 3 weeks, 5β-saturated sterols could no longer be detected in the feces; the fecal neutral sterols consisted entirely of cholesterol plus chow sterols (sitosterol: 57%, campesterol: 14% stigmasterol: 8%,

fucosterol plus 24-ethyl-5α-cholestan-3β-ol: 9%). Both animals developed diarrhea after 3–4 days of antibiotics and lost 25–30% of body weight in 3 weeks. At this time, the percentage of chenodiol in the gallbladder bile from both animals increased to a mean of 74%. In one animal the concentration of ursodiol remained unchanged at 5%, while in the other it decreased from 17% to 11%, and 7-ketolithocholic acid declined in both guinea pigs from a mean value of 35% of total bile acids before treatment to 18% after 3 weeks of antibiotics. Thus, considerable quantities of both ursodiol and 7-ketolithocholic acid persisted in the enterohepatic circulation even after bacterially transformed sterols and bile acids disappeared from the feces.

Three guinea pigs were labeled with [³H]cholesterol immediately after a bile fistula was created. The results from the two control and the one antibiotic-fed animal were the same; chenodiol (CDCA), ursodiol (UDCA), and 7-ketolithocholic acid (7KLCA) were all labeled to a similar specific activity within 1 h after injection (**Fig. 2**).

We infused [³H]ursodiol plus [¹⁴C]chenodiol into another guinea pig approximately 30 min after installing a biliary drain. The results are shown in **Fig. 3** and **Fig. 4**. Almost no ³H was detected in either chenodiol (CDCA) or 7-ketolithocholic acid (7KLCA) over the 6-h duration of the experiment (Fig. 3). However, ursodiol (UDCA) became labeled with ¹⁴C very rapidly and the ¹⁴C specific activities of chenodiol and ursodiol became equal after 1 h (Fig. 4). The labeling of 7-ketolithocholic acid was less efficient, so that the [¹⁴C]7-ketolithocholic acid specific activity never exceeded about 30% of the chenodiol specific activity.

We were also able to identify lathosterol (as 2.5% of total neutral sterols) in the feces of one of the antibiotic-treated animals. While we could detect none of this particular sterol in the chow, we found that the skin sterols of
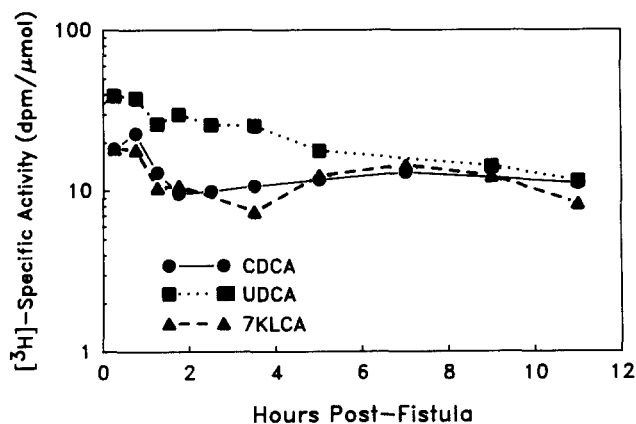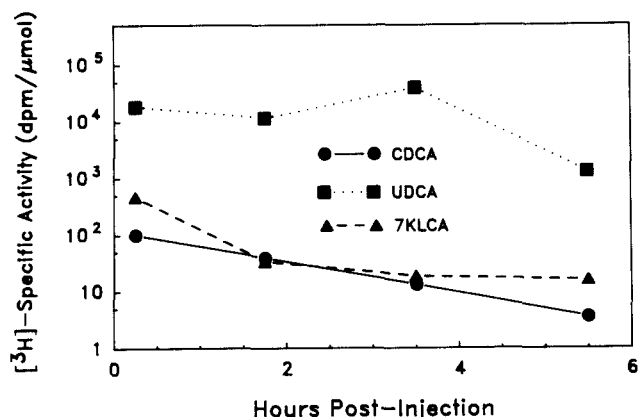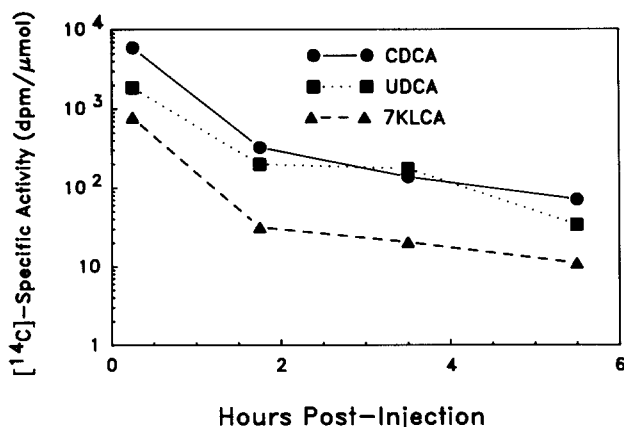

Fig. 3. Time course of the specific activity of chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), and 7-ketolithocholic acid (7KLCA) following the injection of 8.0 μCi of [³H]ursodeoxycholic acid into a bile fistula guinea pig.

the guinea pig consisted of 19% lathosterol and 80% cholesterol.

DISCUSSION

Chenodiol, ursodiol, and 7-ketolithocholic acid became labeled to the same extent when [³H]cholesterol was injected into bile fistula guinea pigs (Fig. 2). This finding demonstrates unequivocally that all three of these bile acids can be made in the liver of this animal. While our conclusions are in conflict with those proposed by earlier investigators (2, 3) they are, most likely, a result of the better analytical techniques that have evolved in the 30 years since the pioneering studies of Danielsson et al. (2). We have recently made the identical observation in the nutria (7).

We have not, as yet, resolved the question of how large a contribution the intestinal bacteria may make to the synthesis of ursodiol and 7-ketolithocholic acid in the nutria (7), but the results of the present study suggest that it may well be small in the guinea pig. Three weeks of antibiotic therapy effectively inactivated much of the bile acid and sterol transforming intestinal bacteria in our guinea pigs (17) as demonstrated by the markedly decreased percentages of unconjugated and monohydroxylated fecal bile acids and 5β-saturated fecal neutral sterols compared to their pre-treatment levels. However, both ursodiol and 7-ketolithocholic acid persisted, in quantity, in the bile. If the major source of ursodiol were 7-ketolithocholic acid made by intestinal bacteria from chenodiol then, as biliary ursodiol decreased moderately in response to the antibiotic, we should have noted a concomitant reduction in the concentration of 7-ketolithocholic acid in the feces. The opposite occurred and the relative concentration of fecal 7-ketolithocholic acid increased by a factor of 2.5. We suggest, therefore, that in

Fig. 2. Time course of the specific activity of chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), and 7-ketolithocholic acid (7KLCA) following the injection of 5.9 μCi of [³H]cholesterol into a bile fistula guinea pig.

Fig. 4. Time course of the specific activity of chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), and 7-ketolithocholic acid (7KLCA) following the injection of 4.5 μCi of [¹⁴C]chenodeoxycholic acid into a bile fistula guinea pig.

the guinea pig as in other animals the intestinal flora are far more likely to degrade 7-ketolithocholic acid, ursodiol, and chenodiol (16) than to oxidize sufficient chenodiol to 7-ketolithocholic acid to produce a 7-ketolithocholic/ursodeoxycholic acid-rich bile. The changes in biliary bile acid composition during antibiotic feeding were probably a consequence of the diarrhea that developed in the animals. Even stronger evidence that the liver itself can synthesize sufficient ursodiol and 7-ketolithocholic acid independent of bacterial intervention comes from the animal that developed a partial biliary obstruction (Fig. 1). At the end of 7 days this animal was synthesizing ursodiol and 7-ketolithocholic acid at a combined rate of 1.2 mg/day which was greater than the rate at which it was making chenodiol. Bile acid synthesis in normal guinea pigs ranges from 4 to 7 mg/day with ursodiol and 7-ketolithocholic acid constituting 30–40% of the total (4–6). Thus, the liver of the guinea pig has sufficient capacity to supply all of the ursodiol and 7-ketolithocholic acid required by the animal each day.

The results of the labeling studies shown in Figs. 3 and 4 suggest that the direction of biosynthesis is from chenodiol to 7-ketolithocholic acid and ursodiol (Fig. 4), as has been suggested (2, 3) and not from ursodiol to chenodiol (Fig. 3). Our observation that the measured specific activity of 7-ketolithocholic acid was much less than that of the ursodiol (Fig. 4) can mean either that 7-ketolithocholic acid is not an important intermediate in the synthesis of ursodiol by the liver or that the biliary and hepatic pools of these bile acids were not in equilibrium during the short time course of the experiment.

In contrast to the guinea pig, previous studies of ours indicated that free chenodiol, ursodiol, and 7-ketolithocholic acid are not interconverted in vivo by the nutria (7). This result might be attributable to species differences in the efficiency with which conjugated and free 7-ketoli-

thocholic acids are transformed to chenodiol and ursodiol (13, 14). Nonetheless, as the partially obstructed guinea pig illustrates (Fig. 1), the nutria and guinea pig may well have similar biosynthetic pathways for chenodiol, ursodiol, and 7-ketolithocholic acid, but with the equilibrium shifted toward chenodiol in the guinea pig and ursodiol in the nutria. The stress of total biliary diversion, or, to a lesser extent, diarrhea and bile acid sequestrants will cause the balance in the guinea pig to tip even further toward chenodiol. ▮▮

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