# An N-acyl glycyltaurine conjugate of deoxycholic acid in the biliary bile acids of the rabbit

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**Abstract** **The biliary bile acid composition of the adult and neonatal domestic rabbit, as well as that of the adult brush rabbit, was characterized. In adult domestic rabbits, the dominant bile acid present was deoxycholic acid (88% of total bile acids), a secondary bile acid formed by the bacterial 7-dehydroxylation of cholic acid. Although most of the bile acids present were conjugated with glycine, two exceptions were observed. About 3% of deoxycholic acid was conjugated, in N-acyl linkage, with glycyl-taurine. Chenodeoxycholic acid, which composed <1% of bile acids, was conjugated solely with taurine. The bile of neonatal rabbits contained a greater percentage of primary bile acids, and bile acids were conjugated to a much greater extent with taurine. The adult brush rabbit had a bile acid composition similar to that of the domestic rabbit, but about one-third of all bile acids were conjugated with taurine. In addition, lithocholic acid was present as its sulfated amidate, whereas in the domestic rabbit, lithocholic acid was conjugated solely with glycine. The biliary bile acid composition of rabbits appears to be unique both in terms of the predominant steroid moiety, as well as in the modes of conjugation.—** Hagey, L. R., C. D. Schteingart, S. S. Rossi, H-T. Ton-Nu, and A. F. Hofmann. **An N-acyl glycyltaurine conjugate of deoxycholic acid in the biliary bile acids of the rabbit. *J. Lipid Res.* 1998.** 39: **2119–2124.**

**Supplementary key words** bile acid metabolism • lithocholic acid • chenodeoxycholic acid metabolism • enterohepatic circulation • bile acid conjugation

Among living mammals, the evolutionary line leading to the domestic rabbit is comparatively ancient; lagomorph ancestors can be traced back to more than 60 million years in the past (1). As part of our continuing investigation of the biliary bile acids of vertebrates in relation to their evolutionary history, we have re-examined the bile acids of the domestic rabbit using high pressure liquid chromatography (HPLC) and mass spectrometry. We report here on the discovery of a dipeptide-conjugated bile acid, identify several bile acids that have not been reported previously in the rabbit, and describe a novel pattern of bile acid conjugation.

## METHODS

### Bile samples

Bile samples were obtained from 7 adult male New Zealand White domestic rabbits, *Oryctolagus cuniculus,* (Simauk, Vista, CA) that had been killed under an approved protocol for unrelated duodenal secretion studies. This project was approved by the animal studies institutional review board. Gallbladder bile was obtained by needle aspiration, diluted in several volumes of reagent grade isopropanol (to prevent bacterial degradation and to precipitate biliary proteins), and stored at 10°C in sealed brown vials. Stillborn neonates were purchased from Simauk's Rabbitry (Vista, CA). The gallbladders were isolated, aspirated, and the contents of 7 animals were pooled. Adult brush rabbit (*Sylvilagus bachmani*) bile was obtained from the Pathology Laboratory of the San Diego Zoo.

### Analytical procedures

Conjugated bile acids were analyzed by HPLC using a modification of the technique of Rossi, Converse, and Hofmann (2). An octadecylsilane column (RP C-18) (Beckman Instruments, Fullerton, CA) was used with isocratic elution at 0.75 ml/min. The eluting solution was composed of a mixture of methanol and 0.01 m $KH_2PO_4$ (67.4% v/v), adjusted to an apparent pH of 5.4 with $H_3PO_4$. Bile acids were quantified by measuring their absorbance at 205 nm. Bile acid amidates (taurine and glycine) have similar extinction coefficients. As dipeptide conjugated bile acids have two N-acyl bonds, their extinction coefficients were calculated by plotting the observed absorbance against injections containing different proportions of known sample weights. Dipeptide-conjugated bile acids show a 1.42 times higher absorbance at 205 nm when compared to the same bile acid conjugated with

Abbreviations: HPLC, higher performance liquid chromatography; RRT, relative retention time; LSIMS, liquid secondary ion mass spectrometry; GC–MS, gas chromatography–mass spectrometry; CDCA, chenodeoxycholic acid; CA, cholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; EEDQ, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline; NMR, nuclear magnetic resonance.

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one N-acyl bond. Bile acids were tentatively identified by matching their relative retention times (RRT) with those of known standards. Relative retention times are calculated in relation to that of chenodeoxycholyl-glycine (RRT = 1.0).

Negative liquid secondary ion mass spectrometry (LSIMS), as well as gas chromatography–mass spectrometry (GC–MS) were used to confirm the HPLC analyses. LSIMS analyses (done at the Bio-organic Biomedical Mass Spectrometry Resource, University of California, San Francisco) were performed using a Kratos VG 70-SE mass spectrometer (Manchester, UK) equipped with a standard VG LSIMS ion source. The instrument was operated at an accelerating voltage of 8 kV and a mass resolution of 1000 (10% valley definition). The liquid matrix was glycerol. For GC–MS, bile acid amidates were deconjugated chemically (1.0 N NaOH, 130°C, 4 h), and the resulting unconjugated bile acids were isolated by solvent extraction into ethyl acetate. They were then analyzed by capillary GC–MS as methyl esters, as methyl ester acetates, or as methyl ester trimethylsilyl derivatives, using a Hewlett-Packard 5890 Gas Chromatograph-5970 MSD, controlled by HP/UX Chem Station software. The column was a Supelco 30 m 0.25 mm ID intermediate polarity SPB-35 (35% phenyl methyl silicone) (Supelco Co., Bellefonte, PA) operated at 275°C (isothermal). A splitless injection was used with an injection temperature of 290°C. Helium was used as the carrier gas with a 7 psi column head pressure. Relative retention times and fragmentation spectra of peaks obtained by GC–MS were compared with those of known standards for identification. Using these techniques, >95% of the total ion current signal in the GC–MS analyses could be assigned to known bile acids and neutral steroids.

The molecular mass of the double conjugates was determined using a Perkin Elmer SCIEX API-3 electrospray triple quadripole mass spectrometer operating in the negative ion mode, with an orifice potential of −60V. Analyses were performed at the Scripps Research Institute Mass Spectrometry Laboratory (La Jolla, CA). The presence of dipeptide conjugates in rabbit bile was also sought using a Finigan LCQ electrospray ion-trap mass spectrometer operated at The Scripps Research Institute Mass Spectrometry Laboratory. The electrospray voltage was 4000 V; the capillary voltage was 30 V.

Proton nuclear magnetic resonance ($^1$H-NMR) spectra were obtained at 500 MHz using a Varian Unity 500 instrument at the Department of Chemistry, University of California, San Diego. The solvent was deuterated methanol, and chemical shifts are expressed in ppm relative to tetramethylsilane.

### Reference compounds

Glycine and taurine conjugates (N-acyl amidates) of chenodeoxycholic acid (CDCA), cholic acid (CA), deoxycholic acid (DCA), and lithocholic acid (LCA) were synthesized by an 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) based method (3). The 3-oxo derivative of DCA was synthesized as previously described (4); sulfo-LCA-taurine and sulfo-LCA-glycine were prepared from LCA-taurine and LCA-glycine using chlorosulfonic acid (5). 12-Epi-deoxycholic acid (also termed "lagodeoxycholic acid") was a gift from Diamalt AG, Munich, Germany and has been characterized previously (6).

Dipeptide conjugates were prepared by coupling the corresponding bile acid glycine conjugates with glycine or taurine using EEDQ (3). The compounds were purified by silica gel chromatography (7) and characterized by $^1$H-NMR and electrospray mass spectrometry.

Deoxycholyl-glycine-glycine: electrospray *m/z* 505; HPLC rrt 1.00; $^1$H-NMR δ 0.698 (s, 3H, Me–18), 0.920 (s, 3H, Me–19), 1.019 (d, J = 6.5 Hz, 3H, Me–21), 3.514 (m, 1H, H–3), 3.872 and 3.876 (2× bs, 4H, −NHC$\underline{H_2}$COOH and −NHC$\underline{H_2}$CONH–), 3.951 (m, 1H, H–12).

Deoxycholy-glycine-taurine: electrospray *m/z* 555; HPLC rrt 0.82; $^1$H-NMR δ 0.697 (s, 3H, Me–18), 0.918 (s, 3H, Me–19), 1.014 (d, J = 6.5 Hz, 3H, Me–21), 2.948 (t, J = 6.5 Hz, 2H, NHCH$_2\underline{CH_2}$SO$_3^-$), 3.508 (m, 1H, H–3), 3.606 (t, J = 6.5 Hz, 2H, NH$\underline{CH_2}$CH$_2$SO$_3^-$), 3.804 (s, −H, NH$\underline{CH_2}$COOH), 3.952 (m, 1H, H–12).

Cholyl-glycine-glycine: electrospray *m/z* 521; HPLC rrt 0.44; $^1$H−NMR δ 0.704 (s, 3H, Me–18), 0.906 (s, 3H, Me–19), 1.031 (d, J = 6.5 Hz, 3H, Me–21), 3.345 (m, 1H, H–3), 3.750 (s, 2H, −NH$\underline{CH_2}$CO–), 3.788 (m, 1H, H–7), 3.869 (s, 2H, −NH$\underline{CH_2}$CO–), 3.948 (m, 1H, H–12).

## RESULTS

Samples of domestic rabbit bile were analyzed by HPLC, and a representative chromatogram is shown in **Fig. 1**, top. Peaks labeled (F) and (K) were readily identified as the common bile acids cholyl-glycine and deoxycholyl-glycine, respectively. Peaks (C), (H), and (M) were identified as 3α-hydroxy-12-oxo-5β-cholan-24-oyl-glycine, 3-oxo-12α-hydroxy-5β-cholan-24-oyl-glycine, lithocholyl-glycine, respectively, based on their known HPLC relative retention times and structural identification using GC–MS.

Using LSIMS, peak **(**B**)** was shown to be a glycine-conjugated dihydroxy bile acid (*m/z* = 448). After deconjugation, formation of the methyl ester, and conversion to TMSi ethers, this bile acid showed a retention time and fragmentation pattern consistent with the structure of 3α,12β-dihydroxy-5β-cholan-24-oic acid. Major fragments included: *m/z* 535 (9%) M–15; 460 (6%) M–90; 370 (30%) M–90–90; 345 (25%) M–90–side chain (SC); 255 (100%) M–90–90–SC; 73 (45%) –Si(CH$_3$)$_3$. The structure of the intact glycine conjugate was confirmed by $^1$H-NMR in d$_4$-methanol. Signal identification was as follows: δ 0.703 (s, 3H, Me–18), 0.943 (s, 3H, Me–19), 1.031 (d, J = 6.5 Hz, 3H, Me–21), 3.338 (dd, J = 4.5 and 11.0 Hz, 1H, H–12), 3.529 (m, 1H, H–3), 3.867 (d, J = 3.0 Hz, 2H, –NH$\underline{CH_2}$COO$^-$).

Peak (G) was shown by LSIMS analysis to be a taurine-conjugated dihydroxy bile acid (*m/z* = 498). After deconjugation, formation of the methyl ester, and conversion to the TMSi ether, this bile acid was found to be the common bile acid CDCA. Its relative retention time on HPLC was also consistent with this bile acid being chenodeoxycholyl-taurine.

HPLC peak (I) was shown by LSIMS analysis to have a molecular mass of *m/z* 555. The difference between this molecular mass and that of a glycine-conjugated dihydroxy bile acid (*m/z* 448) was 97 daltons, equal to the molecular mass of taurine (in an N-acyl linkage). This raised the possibility that peak (I) was a dipeptide conjugate (glycyl-taurine) of a dihydroxy bile acid. After isolation (by TLC) of a further 10 mg of peak (I), a portion was chemically deconjugated, and after derivatization, the bile acid structure was determined by GC-MS. The relative retention time on the column and its fragmentation pattern indicated that this compound was the common bile acid DCA. To confirm the hypothesis that peak (I) was a dipeptide conjugate of DCA, a series of dipeptide conjugates (glycyl-glycine and glycyl-taurine) of DCA and glycyl-glycine of CA were synthesized as described in Methods. These

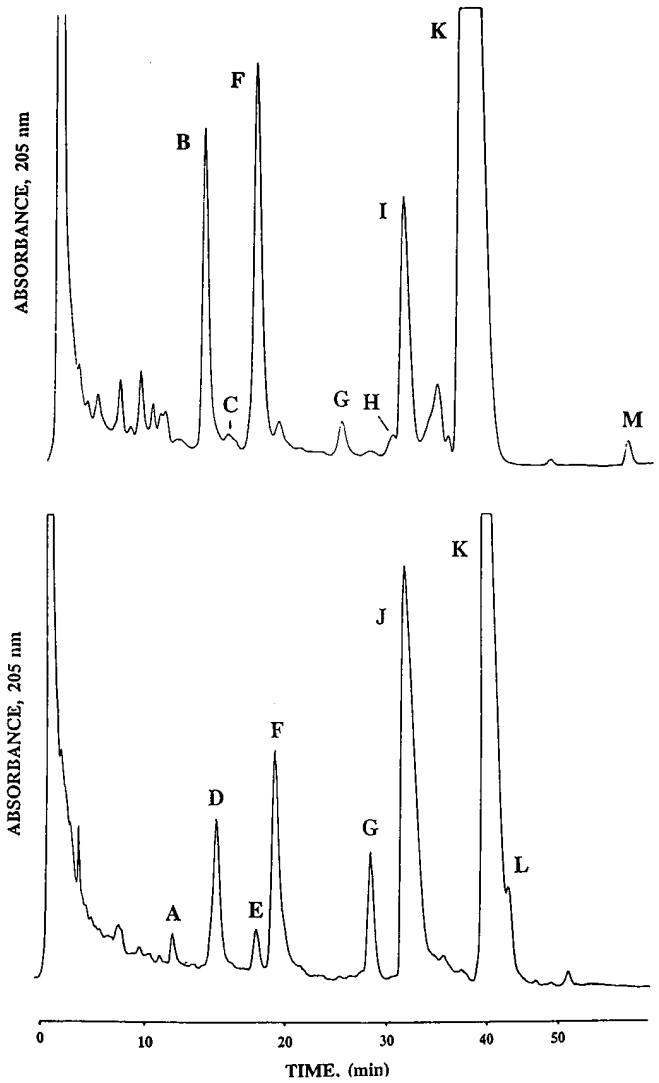


**Fig. 1.** Upper panel: Reversed phase HPLC biliary bile acid profile of a representative domestic rabbit. The relative retention times (to CDCA-glycine) are given in parentheses, and the percent composition follows the semi-systematic name of the compound. (B) 3α,12β-dihydroxy-5β-cholan-24-oyl glycine, 4.2% (0.38); (C) 3α-hydroxy-12-oxo-5β-cholan-24-oyl glycine, 0.1% (0.42); (F) cholyl-glycine, 4.2% (0.48); (G) chenodeoxycholyl-taurine, 0.2% (0.67); (H) 3-oxo-12α-hydroxy-5β-cholan-24-oylglycine, 0.3% (0.80); (I) deoxycholyl-glycine-taurine, 6.1% (0.82); (K) deoxycholyl glycine, 82.2% (1.11); and (M) lithocholyl glycine, 1.1% (2.01). Lower panel: Reversed phase HPLC biliary bile acid profile of a representative brush rabbit. (A) sulfated lithocholyl-taurine 0.4% (0.30); (D) cholyl-taurine, 2.8% (0.37); (E) sulfated lithocholyl-glycine, 0.6% (0.46); (F) cholyl-glycine, 4.9% (0.48); (G) chenodeoxycholyl-taurine, 2.6% (0.67); (J) deoxycholyl-taurine, 28.7% (0.78); (K) deoxycholyl-glycine, 59.5% (1.11); (L) allodeoxycholyl-glycine, 0.5% (1.13).

conjugates, as well as material from peak (I), were then analyzed by $^{1}$H-NMR. The spectrum for HPLC peak (I) was identical with that of the synthetic N-acyl dipeptide conjugate DCA-glycyl-taurine.

Whole rabbit bile was also analyzed by a LCQ electrospray mass spectrometer, as shown in **Fig. 2**. The ion trap detector response was greater for the dipeptide conjugate at *m/z* 555 than for the CA-glycine (at *m/z* 464), a bile acid present in greater concentration. Other potential dipeptide-conjugated metabolites (DCA-glycyl-glyine, *m/z* 505; CA-glycyl-glycine, *m/z* 521) were not present using this technique. The instrument's high sensitivity for dipeptide conjugates suggests that dipeptide conjugates of other bile acids are not present in rabbit bile. A summary of the composition of gallbladder bile acids for seven domestic rabbits by HPLC is given in **Table 1**.

To determine the effect of age on the biliary bile acid composition of the rabbit, samples from 7 stillborn rabbits were collected, pooled, and analyzed. (Table 1). The biliary bile acid composition of the newborn rabbit differed considerably from that of the adult rabbit. The biliary bile acids contained a much greater proportion of primary bile acids (CA, 56% and CDCA, 23%). In addition, each bile acid was about half conjugated with taurine. The presence of taurine-conjugated bile acids in neonatal rabbit bile was confirmed using LSIMS.

The lower panel of Fig. 1 shows the HPLC biliary bile acid profile for a second rabbit species, the Brush rabbit. Peaks labeled (D) and (J) were readily identified as the common bile acids cholyl-taurine, and deoxycholyl-taurine, respectively. Peaks (A), (E), and (L) were identified as sulfolithocholyl-taurine, sulfolithocholyl-glycine, and allodeoxycholyl-glycine, respectively, based on their known HPLC retention times and structural identification using GC–MS. Only one animal was available for analysis and the relative proportions of its bile acids are also included in Table 1. Among the conjugated bile acids, only CDCA was exclusively conjugated with taurine, a finding also found in the domestic rabbit. Deoxycholyl-glycyl-taurine and the oxidoreduction metabolites (3-oxo, 12-oxo, 12-β hydroxy) seen in the domestic rabbit were all absent. In contrast to the domestic rabbit, where most of the bile acids were conjugated predominantly with glycine, about one third of each bile acid was conjugated with taurine in the brush rabbit. In addition, lithocholic acid was present as the sulfate of its glycine or taurine amidate.

## DISCUSSION

These results confirm previous studies (8–10) that the predominant biliary bile acid in the domestic adult rabbit is DCA, a secondary bile acid formed by the bacterial dehydroxylation of CA. Three oxidoreduction metabolites of DCA were also found in small proportion, the 3 oxo-, the 12 oxo-, and the 12β-hydroxy derivatives. The biliary bile acids of the newborn rabbit contained predominantly primary bile acids with a high proportion of CA (56%) and equal parts of CDCA and DCA. The predominant biliary bile acid of the adult brush rabbit was also deoxycholic acid.

In the adult domestic rabbit, DCA was mostly conjugated with glycine. A small proportion was found to be conjugated with the peptide glycyl-taurine. In addition, the small amount of CDCA present was conjugated exclusively with taurine. In the neonatal rabbit, bile acids were conjugated more equally with taurine and glycine. In the

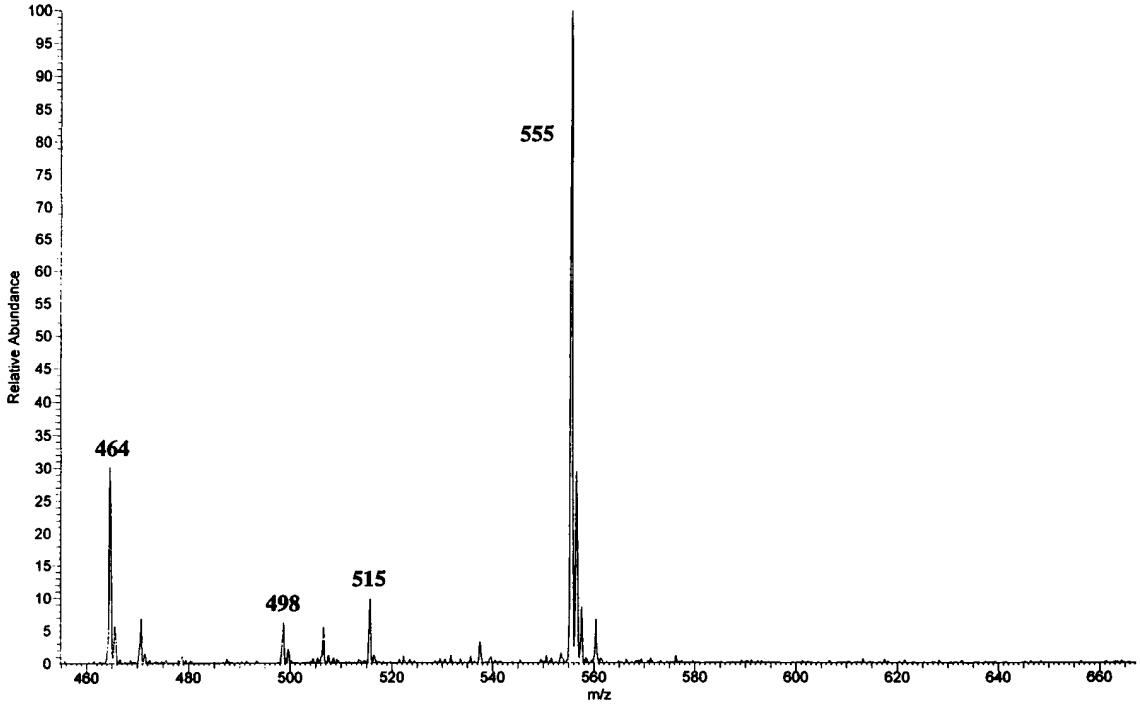


**Fig. 2.** LCQ electrospray ion-trap spectra of whole bile from the domestic rabbit. Peak identification is as follows: *m/z* 464 cholyl-glycine; *m/z* 498 chenodeoxycholyl-taurine; *m/z* 515, a $C_{27}$ tetrahydroxy bile alcohol sulfate; *m/z* 555 deoxycholyl-glycine-taurine.

brush rabbit, bile acids were one-third conjugated with taurine, and LCA was present as sulfated amidates.

**Biliary bile acid composition: steroid moiety**

Rabbits and sperm whales (7) appear to be unique among vertebrates in having a secondary bile acid as their predominant (>75%) biliary bile acid. The rabbit differs from other mammals in having an immense cecum with a capacity ten times greater than that of its stomach (11). In the rabbit, the wet weight of colonic content is 8% of its total body weight, a figure exceeding that of most animals (12). Colonic transit is quite slow (mean residence time 20–56 h) in the rabbit (13). The large lumen, full of anaerobic bacteria, and prolonged retention of colonic contents should provide ample opportunity for DCA to be formed and absorbed.

The fraction of DCA which is absorbed is efficiently conserved during enterohepatic cycling (14), and based on studies in rats (15) and humans (16–18), is likely to be a potent inhibitor of primary bile acid synthesis, thus explaining the dominance of DCA in the circulating bile acids of the rabbit. In addition, the rabbit excretes two types of fecal pellets, one of which is rich in protein and water and one of which is not (19). The rabbit eats the water-rich pellets which are likely to contain DCA, based on the finding of Hellström and Sjövall (14) that this bile acid is found in unconjugated form in rabbit gastric contents. Thus in this species, coprophagy may also contribute to the efficient conservation of DCA.

In the newborn rabbit, deoxycholic acid constituted 21% of biliary bile acids, a value similar to that (23–25%) found by Subbiah et al. (20). As the newborn rabbit is germ-free, the DCA must have originated from placental transfer. Furthermore, because the placenta transports conjugated bile acids vectorially from fetus to mother (21), the DCA in the newborn rabbit is likely to have originated by passive transfer of unconjugated DCA from mother to fetus. The level of unconjugated DCA in adult rabbit plasma is likely to be high, because of absorption of unconjugated DCA from the small and large intestine (22, 23).

In the intestine of the domestic rabbit, CDCA undergoes 7-dehydroxylation to form LCA, a compound present in bile in a proportion twice than of CDCA. Administration of CDCA (or its epimer, UDCA) to the rabbit is known to lead to LCA accumulation and consequent hepatotoxicity, because the rabbit has no means of detoxification of LCA (24). Thus, in the rabbit, 12-hydroxylation in the hepatocyte leads to CA formation and thereby prevents the formation of LCA in the large intestine. We were astonished to find evidence for LCA sulfation in the brush rabbit, as the ability to sulfate LCA and thereby promptly eliminate it from the circulating bile acids has heretofore been reported only in the human (25) and the chimpanzee (26). In principle, the brush rabbit should not develop hepatotoxicity in response to exogenous CDCA or UDCA.

We did not detect 5α bile acids in biliary bile acids, except in the brush rabbit. Allocholic acid has been described in both the adult rabbit (27) as well as in the infant germ-free domestic rabbit (28). Allocholic acid may be formed by bacteria from DCA, indicating that it may be a secondary bile acid. However, its presence in the infant germ-free domestic rabbit indicates that it may also be a primary bile acid.

TABLE 1. HPLC analysis of rabbit gallbladder bile acids[a]

| Steroid Moiety[b] | Domestic Rabbit Adult[c] | Domestic Rabbit Fetal[d] | Brush Rabbit |
|---|---|---|---|
| | | % | |
| Cholic acid family | | | |
| 3α,7α,12α (CA) | 3.7 ± 1.2 | 56.2 | 7.7 |
| 3α,12α | 88.5 ± 2.3 | 21.1 | 88.2 |
| Allo-3α,12α | 0 | 0 | 0.5 |
| 3α,12β | 5.3 ± 1.4 | 0 | 0 |
| 3α,12-oxo | 0.4 ± 0.3 | 0 | 0 |
| 3-oxo, 12α | 0.2 ± 0.1 | 0 | 0 |
| Chenodeoxycholic acid family | | | |
| 3α,7α (CDCA) | 0.7 ± 0.5 | 22.7 | 2.6 |
| 3α (LCA) | 1.2 ± 0.3 | 0 | 1.0 |
| Mode of conjunction | CA | DCA | CDC |
| Adult | | % | |
| Glycine | 100 | 97.5 | 0 |
| Taurine | 0 | 0 | 100 |
| Gly-Tau[e] | 0 | 2.5 | 0 |
| Fetal | | | |
| Glycine | 54.5 | 60.1 | 66.9 |
| Taurine | 45.6 | 39.9 | 33.1 |
| Gly-Tau | 0 | 0 | 0 |
| Brush | | | |
| Glycine | 63.7 | 67.5 | 0 |
| Taurine | 36.3 | 32.5 | 100 |
| Gly-Tau | 0 | 0 | 0 |

[a] Bile acid composition has been normalized to 100%.
[b] All bile acids are 5β-C24 acids unless otherwise noted. The positions of nuclear substituents are indicated.
[c] Values from 7 individuals are expressed as mean ± standard deviation.
[d] Bile samples from 7 individuals were pooled.
[e] The value for this compound was corrected for a 1.42× higher absorbance at 204 nm (due to the presence of an additional peptide bond).

### Biliary bile acid composition: conjugation

A glycyltaurine conjugate of DCA was identified. Dipeptide conjugates of xeno- and endobiotics have also been reported in other vertebrates (29), but natural dipeptide conjugates of bile acids have not been observed previously. In the crab, a sarcosyltaurine conjugate of dodecanoic acid is the major digestive surfactant secreted by the hepatopancreas (30). In our laboratory, dipeptide conjugates have not been detected in analyses of bile from several hundred species, suggesting that they rarely occur in nature.

In most species that conjugate with both glycine and taurine, the pattern of conjugation of individual bile acids is identical (31). However, in both the domestic and brush rabbits, CDCA was conjugated solely with taurine. We were astonished to find taurine-conjugated CDCA in an otherwise all-glycine-conjugating animal, and although the amount of taurine-conjugated CDCA was low (0.7% in the domestic rabbit), no corresponding amount of glycine-conjugated CDCA could be found, despite an extensive search.

Conjugation of bile acids with taurine as well as with glycine was observed in the newborn rabbit, in agreement with the report of Subbiah et al. (20). There may be functional advantages in conjugating bile acids with taurine rather than with glycine in the fetal and newborn animal (32). At acidic pH, taurine conjugates are far more water-soluble and membrane-impermeable than glycine conjugates (33). In the proximal small intestine, such properties would promote a high intraluminal concentration, thereby enhancing lipid absorption in the nursing infant. In addition, for a given steroid moiety, taurine-conjugated bile acids are less cytotoxic (34, 35), and more resistant to bacterial deconjugation (36). Tammar (37) analyzed bile from the related Black-tailed jack rabbit (*Lepus californicus*), and noted that both taurine and glycine conjugates were present.

The human bile acid-CoA:amino acid N-acyltransferase has recently been cloned, and studies of the expressed protein indicate that it can transfer the bile acid CoA thioester to either glycine or taurine (38). A second N-acyl transferase, cloned from mouse liver and similar in structure to the human enzyme, has been found to be taurine specific (39). The finding that CDCA is conjugated solely with taurine in the midst of a dominant pattern of glycine conjugation raises the possibility that two enzymes are involved in bile acid conjugation. Which enzyme is involved in the conjugation of deoxycholyl-glycine with taurine is not known.

In conclusion, the biliary bile acids of the rabbit appear to be unique among mammals in consisting predominantly of deoxycholic acid, a secondary bile acid. The conjugation pattern of biliary bile acids in the rabbit is also unique in that a glycyltaurine conjugate has been identified, as well as the presence of a primary bile acid conjugated exclusively with taurine in an animal where bile acids are mostly conjugated with glycine.

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