The role of conjugation reactions in enhancing biliary secretion of bile acids

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Shortening the five-carbon carboxylic acid side chain of cholic acid by one methylene group gave rise to a bile acid (norcholeate) that was not a substrate for the bile acid-conjugating enzymes. The metabolism and biliary secretion of norcholeate in intact liver was examined in the isolated perfused rat liver system. When rat livers were perfused with 14–20 μ M solutions of norcholeate for 10 min, norcholeate was found in the unconjugated form in liver, venous effluent and bile. Neither tauronorcholeate nor glyconorcholeate was detectable by high-pressure liquid chromatography or fast-atom-bombardment mass spectrometry. The kinetics of hepatic uptake and biliary secretion of norcholeate was compared with that for cholate, taurocholate and chemically synthesized tauronorcholeate. The latter three bile acids were completely cleared from the perfusate and efficiently secreted into the bile. However, norcholeate was incompletely extracted from the perfusate, and this was shown to be at least partially due to its relatively lower rate of hepatic uptake. Furthermore, the rate of norcholeate secretion into bile was greatly reduced relative to the secretion of cholate or chemically synthesized tauronorcholeate, even though the concentration of norcholeate in the liver was comparatively high. These data demonstrate that the conjugation of bile acids greatly facilitates their secretion into bile.

Bile acids are conjugated in the liver with glycine or taurine before their secretion into bile. In an extensive survey of bile composition in nearly 100 species, Haslewood (1967) reported appreciable amounts of unconjugated bile acids only in the bile of amphibians. In higher vertebrates, it appears that either unconjugated bile acids cannot efficiently cross the canalicular membrane or that conjugation reactions are so efficient that conjugation cannot be avoided. O'Maille *et al.* (1965) infused cholic acid into dogs and found unconjugated cholate in bile but only after the liver had been depleted of taurine by continuous infusion with a high concentration of cholate (>2 μ mol/kg body wt. per min) for several hours. Structural alterations of the bile canaliculi occur under these conditions (Layden & Boyer, 1978) and this could account for the appearance of cholate in the bile.

Our approach to determining the extent to which conjugation enhances the rate of bile-salt secretion into bile has been to study the behaviour of a bile

acid that cannot be conjugated *in vivo*, but which is structurally and ionically analogous to naturally occurring bile acids. Recent work has revealed that shortening the five-carbon carboxylic acid side chain of cholic acid by one methylene unit leads to a bile acid (norcholeate) that cannot be conjugated (Czuba & Vessey, 1982). The present study employs an isolated perfused rat liver system to compare the kinetics of biliary secretion of norcholeate and chemically synthesized tauronorcholeate, and contrasts these data with those obtained for cholate and taurocholate.

Materials and methods

Materials

Cholic acid (99–100% pure) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and was recrystallized as described previously (Vessey, 1979). Sodium taurocholate (more than 99% pure) was obtained from P-L Biochemicals Inc., Milwaukee, WI, U.S.A. Norcholic acid (3 α ,7 α ,12 α -trihydroxy-23-nor-5 β -cholanic acid) was purchased from Research Plus Inc., Bayonne, NJ, U.S.A., and

Abbreviations used: f.a.b., fast atom bombardment; h.p.l.c., high-pressure liquid chromatography.

was found to be impure by t.l.c. on silica gel G developed with chloroform/methanol (7:3, v/v). Norcholate was purified on a column of Bio Sil A (Bio-Rad Laboratories, Richmond, CA, U.S.A.) equilibrated with chloroform. Norcholate was applied in chloroform/methanol (14:3, v/v) and the column was eluted with the same mixed solvent. The contaminant was eluted from the column before norcholate. The structure and purity of norcholate was confirmed by g.l.c.-mass spectrometry and by f.a.b. mass spectrometry. Taurocholate was synthesized from the purified norcholate by the method of Tserng *et al.* (1977) and its structure and purity verified by h.p.l.c. and f.a.b.-mass spectrometry. Fluosol-43 was obtained from Alpha Therapeutic Corp., Los Angeles, CA, U.S.A. [^3H]CoA (sp. radioactivity 1 Ci/mmol) was obtained from New England Nuclear Corp., Boston, MA, U.S.A. SEP-Pak C 18 cartridges were obtained from Waters Associates, Milford, MA, U.S.A.

Methods

Enzymic assays. Bile acid-CoA:glycine/taurine *N*-acyltransferase activity was assayed as described previously (Czuba & Vessey, 1980). Microsomal bile acid-CoA synthetase activity was determined with a radioassay employing [^3H]CoA. Assays contained 3 mM-Mg $^{2+}$, 3 mM-ATP, 50 mM-Tris, pH 7.6, 50 μM -[^3H]CoA, 1–2 mg of microsomal protein per ml, and the indicated concentration of bile acid. Reactions were stopped by adding EDTA to a concentration of 10 mM. The samples were placed in a boiling-water bath for 30 s and then denatured protein was removed by centrifugation. The supernatants were spotted on Adsorbosil-Plus 1 t.l.c. plates (Applied Science Labs., State College, PA, U.S.A.). The plates were developed in butanol/acetic acid/water (20:15:1, by vol.). Bile acid-CoA adducts migrate approximately one-third of the way up the plate, whereas CoA (oxidized and reduced) stays at the origin. The zone containing the bile acid-CoA adduct was scraped from the plate and counted in a Beckman LS-150 liquid-scintillation counter (36% counting efficiency).

Isolated perfused liver. Isolated rat livers were perfused initially with a haemoglobin-free medium consisting of a Krebs–Henseleit buffer and a perfluorochemical oxygen carrier in a Krebs–Ringer bicarbonate solution with 0.2% glucose (Fluosol-43). The perfusion system and viability of the liver preparation has been documented previously (Gollan *et al.*, 1981). Male Sprague–Dawley rats weighing 240–260 g were used as liver donors. Each liver was perfused via the portal vein at a rate of 25 ml per min for 60 min, with a recirculating Fluosol solution containing 0.2 mM-aurine, adjusted to a pH of 7.4, and oxygenated with 100% O $_2$. Bile was collected continuously for 10 min periods

during this time and analysed for total bile acid content. This was followed by a 3 min, single-pass perfusion with Krebs–Ringer bicarbonate solution (Dawson & Elliott, 1959) containing 0.2% glucose and 0.2 mM-aurine that removed the Fluosol from the system. The liver was then perfused single-pass for 10 min at 25 ml per min with the test solution which contained 14–20 μM bile acid in Krebs–Ringer/taurine solution adjusted to pH 7.4 and oxygenated with O $_2$ /CO $_2$ (95:5). Venous effluent was sampled continuously as 10 s collections and bile flow was calculated from the time taken to produce 10 μl of bile. At the termination of each experiment, the liver was removed, firmly blotted to remove excess fluid, quick-frozen and stored at -50°C for subsequent analysis.

Bile acid analysis. Total bile acid content in test solutions, in venous effluent and in bile samples was determined by enzymic spectrofluorimetry (Osuga *et al.*, 1977) using 3 α -hydroxy-steroid dehydrogenase coupled with Diaphorase (Accurate Chemical & Scientific, Westbury, NY, U.S.A.) (Haslewood, 1967). To analyse for specific bile acids in test solutions, venous effluent and bile were extracted quantitatively on Sep-Pak C 18 cartridges (Whitney & Thaler, 1980). Liver homogenates were refluxed in alkaline ethanol for 30 min, filtered through glass-fibre paper, dried under vacuum, suspended in 0.1 M-sodium acetate buffer, pH 5.6, and re-extracted on Sep-Pak C 18 cartridges (Whitney & Thaler, 1980). High-pressure liquid chromatography was carried out on a Waters radial compression (RC) separation module, using a 10- μ particulate C 18 cartridge (0.5 cm $\times$ 10.0 cm) and methanol/water/acetic acid (75:25:4, by vol.), pH 5.0, as mobile phase (Whitney *et al.*, 1980). The flow rate was 2.5 ml/min and detection was by refractometry. Fractions corresponding to standard bile salts were collected and re-extracted on Sep-Pak C 18 cartridges.

For g.l.c., extracts and h.p.l.c. fractions were pressure-hydrolysed in 15% NaOH in methanol [4 h at 15 psi (103.5 kPa)]. The methyl ester of the carboxy function and the trimethylsilyl ethers of the hydroxy groups were prepared by reaction with diazomethane followed by treatment with hexamethyldisilazane/trimethylchlorosilane/pyridine (3:1:9, by vol.) (Grundy *et al.*, 1965). Glass-capillary g.l.c. was performed on 25 m, wall-coated, open tubular glass capillaries (0.25 mm internal diameter) coated with Carbowax 20M. A Varian 600 gas chromatograph equipped with a flame-ionization detector and a Vista 401 data system was used for quantification. 23-Nordeoxycholic acid was added as an internal standard before derivative formation, and the column was programmed from 24 to 60 $^\circ\text{C}$ at 5 $^\circ\text{C}$ per min. G.l.c.-mass spectrometry was carried out using the same column coupled to a Kratos-MS-25

medium-resolution mass spectrometer (Whitney *et al.*, 1980). Mass spectrometry of unchanged, intact, conjugated, and non-conjugated bile salts was performed on a Kratos-MS-50 mass spectrometer, using an f.a.b. ion source equipped with a Saddlefield ion gun to produce a neutral atom beam (intensity 40–60 mA; energy range approx. 6 kV) (Whitney & Burlingame, 1982). Glycerol was applied to the probe as a proton source.

Results

Studies *in vitro* of the substrate specificity of the enzyme bile acid CoA:glycine/taurine *N*-acyltransferase purified from bovine liver revealed that the enzyme cannot use norcholyl-CoA as a substrate (Czuba & Vessey, 1982). Because the perfusion experiments reported in the present paper utilized rat liver, we have examined the substrate specificity of a partially purified preparation of *N*-acyltransferase from rat liver. At 37°C, 50 μ M-norcholyl-CoA and 50 mM-taurine, there was no detectable rate of reaction. Reaction of taurine with cholyl-CoA under similar conditions was rapid. Based on the limits of detection, it can be stated that the rate of reaction with norcholyl-CoA was less than 1% of the rate of reaction with cholyl-CoA.

A similar study was conducted on the other enzyme of bile acid conjugation, that is, the activating enzyme bile acid-CoA synthetase. The activation of norcholate by a partially purified preparation of enzyme from bovine liver was undetectable. Thus, over a short-term perfusion (less than 10 min), one would expect norcholate not to be metabolized by rat liver.

The enzymic studies *in vitro* suggest that norcholate cannot be conjugated *in vivo*. To verify this and determine the kinetics of plasma clearance and biliary secretion of this 'non-conjugable' bile acid, we utilized an isolated perfused rat liver system (see the Materials and methods section for details). Rat livers ($n = 6$) were perfused for 60 min with 50 ml of recirculating Fluosol solution containing 0.2 mM-taurine in order to deplete the existing bile acid pool. During this period, the bile acid concentration in bile fell from approx. 25 mM to 1–2 mM, and the mean rate of bile flow decreased from 12 μ l per min to 6–7 μ l per min. When the Fluosol solution was washed out with a 3 min period of single-pass perfusion with 0.2 mM-taurine in Krebs solution, the concentration of bile acid in the bile was always less than 1 mM. Perfusion was then initiated with the test solution containing either 14 μ M- or 20 μ M-norcholate in Krebs solution containing 0.2 mM-taurine. The venous perfusate and bile were collected continuously over the 10 min test period for analysis.

Results of the analysis performed on venous samples collected during the 10 min perfusion period

Fig. 1. Concentration of norcholate in the perfusion solution and in the venous effluent as a function of the time course of perfusion

A rat liver was perfused with a 14 μ M solution of norcholate in Krebs–Ringer bicarbonate/taurine, as described in the Materials and methods section. This is referred to as the arterial concentration of norcholate (----). The venous effluent from the liver was collected at the indicated time points and the concentration of norcholate (□) determined enzymically (see the Materials and methods section).

with 14 μ M-norcholate are shown in Fig. 1. The concentration of bile acid in the venous perfusate increased to a mean value of 3.5 ± 1.3 μ M during the initial 90 s and thereafter remained relatively constant. The drop in norcholate concentration from 14 μ M on the arterial side of the liver to 3.5 μ M on the venous side indicates that approx. 75% of the norcholate was cleared in a single pass. When the liver was perfused with a higher concentration (20 μ M) of norcholate, venous concentrations as high as 8 μ M were obtained. Thus, only 60% of the norcholate was cleared at the higher concentrations. On the other hand, the liver was able to clear 100% of a 14 μ M solution of tauronorcholate.

Analyses of the bile samples obtained in the perfusions with 14 μ M-norcholate and with 14 μ M-tauronorcholate for total bile acid are shown in Fig. 2. For tauronorcholate, there was a 2–3 min lag, followed by a rapid increase in bile acid concentration over a period of 5 min to a mean value of 28.4 ± 2.1 mM. In contrast, liver perfusion with norcholate was characterized by an initial 4–5 min lag and then a gradual increase over the next 5–6 min to a mean concentration of 7.7 ± 1.28 mM. Analysis by h.p.l.c. of the bile samples from the norcholate perfusion (see the Materials and methods section) revealed 80% unconjugated norcholate and 20% taurocholate. The identification of these two bile acids was confirmed by f.a.b.-mass spectrometry (see the Materials and methods section). Analysis of bile from the tauronorcholate perfusion experiments revealed approx. 90% tauronorcholate, 7% taurocholate and a trace amount of norcholate.

Fig. 2. Time course for the appearance of norcholeate or tauronorcholeate in bile

A rat liver was perfused with either 14 μM-norcholeate (hatched columns) or 14 μM-tauronorcholeate (open columns). Bile was collected continuously in 10 μl increments and analysed for 3 β-hydroxy-steroid, as described in the Materials and methods section.

In additional experiments, livers were perfused with cholate ($n=3$) or taurocholate ($n=1$), and in both cases, the results were virtually identical with those obtained with tauronorcholeate. The single-pass hepatic clearance of cholate and taurocholate was complete. With a perfusion concentration of 14 μM for either bile acid, the concentration of bile acid in bile reached a plateau of approx. 30 mM after 10 min of perfusion. H.p.l.c. analysis of the bile after taurocholate perfusion revealed only taurocholate. However, bile from the cholate perfusions contained about 93% taurocholate and 6% glycocholate. Unconjugated cholate was not detectable.

At the conclusion of the norcholeate and tauronorcholeate perfusions, the livers were firmly blotted with gauze to remove excess liquid, and analysed for bile acid content by quantitative g.l.c. after hydrolysis to free bile acids and derivative formation (Table 1). Unhydrolysed samples were analysed by h.p.l.c. (Table 2). Table 1 shows that perfusion with norcholeate resulted in a comparatively high concentration of norcholeate in the liver (184 ± 25 μg/g) and, as indicated in Table 2, all of this is in the unconjugated form. By comparison, perfusion with tauronorcholeate resulted in a hepatic tauronorcholeate concentration of less than 83 ± 16 μg/g.

Discussion

The shortening of the five-carbon side chain of cholic acid by one methylene group (norcholic acid) results in a bile acid structure that cannot be conjugated with glycine or taurine *in vitro* (Czuba &

Table 1. Bile acid analysis of hydrolysed liver extracts by glass capillary g.l.c.

Identification of the bile acids was confirmed by g.l.c.-mass spectrometry. The number of livers analysed is indicated by the value of n and for norcholeate and tauronorcholeate the data are expressed as means $\pm$ S.D.

Perfusate	Bile acid concn. (μg/g wet wt. of liver)						Norcholeate
	Cholate	Deoxycholate	Chenodeoxycholate	β-Muricholate	Lithocholate	γ-Muricholate	
Norcholeate ($n=4$)	17 ± 6.6	3.5 ± 2.6	1.6 ± 1.2	24 ± 17	0	46 ± 22	184 ± 25
Tauronorcholeate ($n=4$)	21 ± 9.6	trace	trace	23 ± 4.8	0	24 ± 15	83 ± 16

Table 2. Bile acid analysis of unhydrolysed liver extracts by radial compression high-pressure liquid chromatography. Liver extracts were analysed by high-pressure liquid chromatography, as described in the Materials and methods section. Identification of the bile acids was confirmed by f.a.b.-mass spectrometry. The numbers in the Tables are expressed as the mean percentage standard deviation of the total bile acid concentration represented by the respective bile acid. The column labelled 'Other' is a sum of the concentrations of β- and γ-tauromuricholate, taurochenodeoxycholate, taurodeoxycholate and glycocholate. The number of livers analysed is indicated by n . Results are means $\pm$ S.D.

Perfusate	Cholate (%)	Taurocholate (%)	Norcholeate (%)	Tauronorcholeate (%)	Other (%)
Norcholeate ($n=4$)	—	7.9 ± 4.7	72.9 ± 8.9	—	18.9 ± 8.1
Tauronorcholeate ($n=4$)	—	8.1 ± 2.5	—	67.7 ± 8.8	24.0 ± 7.6

Vessey, 1982). In the present investigation, we studied the metabolism of norchole in an isolated perfused rat liver system. Norchole was found in the unconjugated form in the liver, venous effluent and bile. Neither tauronorchole nor glyconorchole was detectable by h.p.l.c. or f.a.b.-mass spectrometry of liver extracts and bile. Based on these findings, we conclude that norchole cannot be conjugated by rat liver *in vivo*.

The inability of rat liver to conjugate norchole provided a useful tool for determining the extent to which conjugation affected biliary secretion. The hepatic uptake and biliary secretion of norchole was compared with that of chemically synthesized tauronorchole in the perfused rat liver. Hepatic extraction of norchole from the perfusate was greatly reduced relative to tauronorchole. Tauronorchole was completely cleared from the perfusate in a single pass, whereas norchole was incompletely extracted from the perfusate (Fig. 1). The presence of norchole in the venous effluent was due, at least in part, to decreased hepatic uptake relative to that of tauronorchole. If the norchole in the venous perfusate was attributable to the fact that norchole taken up by the liver accumulates and then refluxes back into the perfusate, then it would be expected that the initial venous samples taken before the system achieved steady state would reflect complete extraction. In fact, the first 10 s sample of venous perfusate contained over 1 μ M-norchole and the data for the early time points give no indication of extrapolating back to zero. On the other hand, the concentration of norchole in the venous perfusate does increase during the initial 1–2 min time intervals and this may be due to reflux of norchole back into the perfusate as saturation of the hepatic pool of norchole occurs. The basis for this accumulation of norchole is revealed by the pattern of bile acid excretion into bile (Fig. 2). Norchole has a greatly reduced rate of biliary excretion relative to that of tauronorchole, taurochole or chole. Consequently, the total hepatic bile acid concentration was elevated in the norchole perfusion as compared with that with tauronorchole (Table 1), taurochole or chole (D. A. Vessey, J. Whitney & J. L. Gollan, unpublished work). Moreover, the rate of norchole secretion into bile was low despite the fact that the hepatic concentration of norchole is relatively high. The mean hepatic concentration of

norchole in the liver was 184 μ g/g of liver (0.49 μ mol/g of liver), whereas in the livers perfused with tauronorchole, the total bile acid concentration was only 151 μ g/g of liver and the mean tauronorchole concentration was only 83 μ g/g of liver. Thus, norchole is inefficiently secreted into bile even at hepatic concentrations greatly exceeding those at which conjugated bile acid is readily secreted. The demonstration that the chemically synthesized conjugate of norchole is much more effectively secreted in the bile than unconjugated norchole strongly suggests that conjugation is important for the rapid secretion of bile acids.

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