Bile Salts of the Green Turtle *Chelonia mydas* (L.)

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1. Bile salts of the green turtle *Chelonia mydas* (L.) were analysed as completely as possible.
2. They consist of taurine conjugates of $3\alpha,7\alpha,12\alpha,22\xi$ -tetrahydroxy- 5β -cholestan-26-oic acid (tetrahydroxysterchocholic acid) and $3\alpha,12\alpha,22\xi$ -trihydroxy- 5β -cholestan-26-oic acid, with minor amounts of $3\alpha,7\alpha,12\alpha$ -trihydroxy- 5β -cholan-24-oic acid (cholic acid), $3\alpha,12\alpha$ -dihydroxy- 5β -cholan-24-oic acid (deoxycholic acid) and possibly other bile acids.
3. Cholic acid and deoxycholic acid represent the first known examples of bile acids common to chelonians and other animal forms: they may indicate independent evolution in chelonians to C_{24} bile acids.
4. The discovery of a 7-deoxy C_{27} bile acid is the first evidence that C_{27} bile acids or their conjugates have an enterohepatic circulation.

Reptilian bile salts, like those of all vertebrates above the level of organization of amphibians, are conjugates of bile acids, and conjugation is with taurine. Chelonians (tortoises and turtles) arose from reptilian stock that has been distinguishable from the remainder since the discovery of the earliest fossil reptiles. As reptiles, they are both primitive and, of course, highly specialized. In conformity with their history, chelonian bile salts have not until now been found to include amphiphilic steroid substances in common with those of any other group of animals.

Workers in Japan (see Tammar, 1974) were the first to show that a characteristic chelonian bile acid is tetrahydroxysterchocholic acid ($3\alpha,7\alpha,12\alpha,22\xi$ -tetrahydroxy- 5β -cholestan-26-oic acid; Ia), which readily forms a lactone (IIa). It has been clear for

some time that other acids are also present in chelonians (Tammar, 1974). Tammar (1970) separated ethylated bile acids from the green turtle *Chelonia mydas* on Al_2O_3 : he accounted for about 50% of the separated material as compound (Ia) and characterized ethyl tetrahydroxysterchocholate (Ic).

We have re-examined bile salts of *Chelonia mydas*, collected in conditions excluding changes *post mortem*, by techniques offering greater insight than methods previously used.

Materials and Methods

General methodology was that of Anderson *et al.* (1974) or Amos *et al.* (1977), except where otherwise stated. G.l.c.–mass-spectroscopy conditions were the

- (Ia) $R^1 = OH, R^2 = H$
 (Ib) $R^1 = OH, R^2 = CH_3$
 (Ic) $R^1 = OH, R^2 = C_2H_5$
 (Id) $R^1 = H, R^2 = CH_3$
 (Ie) $R^1 = R^2 = H$

- (IIa) $R^1 = OH$
 (IIb) $R^1 = H$

same as reported by Anderson *et al.* (1974), except that the g.l.c. column (length 123cm) was of 2% SP-525 on 100/120 Supelcoport (Supelco, Bellefonte, PA 16823, U.S.A.) and was heated to 220–245°C. N.m.r. measurements were carried out on a Bruker WH-90 Fourier-transform spectrometer (Bruker Scientific, Palo Alto, CA U.S.A.) at 90 MHz with tetramethylsilane as internal reference and solvent pentadeuteropyridine with or without a drop of $^2\text{H}_2\text{O}$.

Bile (preserved in ethanol) from *Chelonia mydas* was obtained through Professor Sir Alan Parkes and Professor E. C. Amoroso as part of an investigation of turtle farming on Grand Cayman Island. Weights of bile salts, isolated and partially purified as described by Haslewood (1967), from eight turtles ranged from 0.673 to 1.677 g and colour from very dark-green to light-brown. Preliminary t.l.c. in the systems (1) acetic acid/3-methylbutyl acetate/water (5:5:2, by vol.) or (2) chloroform/ethanol/ethyl acetate/acetic acid/water (8:6:5:4:1, by vol.) gave nearly identical results with every specimen and showed at least four components of the bile salts. No discrete spot corresponded to the taurine conjugate of cholic acid ($3\alpha,7\alpha,12\alpha$ -trihydroxy-5 β -cholan-24-oic acid) and one spot had almost the same R_F as the chief bile salt of the frog *Discoglossus pictus*, which is the taurine conjugate of trihydroxycoprostanic acid ($3\alpha,7\alpha,12\alpha$ -trihydroxy-5 β -cholestan-26-oic acid; Anderson *et al.*, 1974). Further purification was as follows.

Preparative t.l.c. of bile salts

Plates (20cm $\times$ 20cm) coated with Silicagel H (0.25mm thick, from E. Merck A.-G., Darmstadt, Germany) were loaded with bile salts (about 2.7mg per plate) as a thin band. After chromatography in system (2), four zones (A, B, C, D in order of increasing R_F) were revealed in I_2 vapour, scraped off and eluted with methanol (2.5 ml per zone). The combined products from eight plates were purified on Sephadex LH-20 as described by Haslewood & Haslewood (1972) and examined by i.r. spectroscopy.

Separation of hydrolysed bile salts

Bile salts or samples (8.7–20mg, from 11 plates) of material obtained from the four separated zones (A–D) mentioned above were heated with 1M-NaOH (8ml) in a sealed metal bomb at 118°C for 17h. The products were diluted with water, acidified with 6M-HCl and extracted with ethyl acetate (2 $\times$ 10ml). The combined ethyl acetate extracts were washed with water, dried over Na_2SO_4 and evaporated. Approximate percentages by weight of bile acids so obtained from zones A–D were as follows: A, 42.0; B, 42.0; C, 7.5; D, 9.2. The bile acids, in methanol, were methylated with diazomethane and the methyl esters analysed by t.l.c. in the system (3) benzene/propan-2-ol/acetic acid (15:5:1, by vol.).

Methylated bile acids from zones A and B gave two spots and were further purified as described for bile salts on Silicagel G (Merck) in system (3). The products from zone A, after further purification as described by Ikawa (1976), were chromatographically identical with products formed after hydrolysis and subsequent methylation of a sample of ethyl tetrahydroxysterchohanate (Ic) kindly supplied by Dr. A. R. Tammar.

Methylated bile acids from zones C and D showed a single main spot on chromatography and were further analysed by g.l.c., g.l.c.–mass spectroscopy and n.m.r. spectroscopy. A sample of the total bile salts, hydrolysed and methylated as described, was analysed by g.l.c.–mass spectroscopy and the results were compared with those from methylated bile acids from the separated zones A–D.

Results

(In the following, letters A–G refer to mass-spectral cleavage fragments indicated in formulae I or II: M = molecular mass. For fragment C, $\text{R}^2 = \text{CH}_3$ or C_2H_5).

I.r. spectra of the bile salts showed that they are taurine conjugates of bile acids, most of which have the substituted ring nucleus of cholic acid. Methyl esters of bile acids obtained after hydrolysis of material eluted from zone A (R_F 0.03–0.16) of the preparative-t.l.c. separation of the bile salts consisted of two compounds (a) and (b), both of which had an i.r. spectrum clearly indicating the substituted ring nucleus of cholic acid. The same compounds were formed after hydrolysis and methylation of ethyl tetrahydroxysterchohanate (Ic).

Compound (a), less polar on t.l.c. in the systems used, gave, as its trimethylsilyl (Me_3Si) derivative, mass-spectral peaks at m/e 574 ($M-\text{Me}_3\text{SiOH}$) $^+$, 559 ($M-\text{Me}_3\text{SiOH}-\text{CH}_3$) $^+$, 484 ($M-2\text{Me}_3\text{SiOH}$) $^+$, 469 ($M-2\text{Me}_3\text{SiOH}-\text{CH}_3$) $^+$, 394 ($M-3\text{Me}_3\text{SiOH}$) $^+$, 343 ($E-2\text{Me}_3\text{SiOH}$) $^+$, 281 ($F-3\text{Me}_3\text{SiOH}$) $^+$, 253 ($E-3\text{Me}_3\text{SiOH}$) $^+$ and 113 (G) $^+$, consistent with its formulation as the lactone (IIa) of tetrahydroxysterchohanic acid. Its n.m.r. characteristics are shown in Table 1.

The more polar *compound (b)* was obtained as white needles, m.p. 99–101°C, from aq. methanol; its Me_3Si derivative exhibited in its mass spectrum a very intense peak at m/e 217 (C) $^+$ and small peaks at m/e 383 ($D-3\text{Me}_3\text{SiOH}$) $^+$, 293 ($D-4\text{Me}_3\text{SiOH}$) $^+$, 253 ($A-3\text{Me}_3\text{SiOH}$) $^+$, 185 ($C-\text{CH}_3\text{OH}$) $^+$ and 127 ($C-\text{Me}_3\text{SiOH}$) $^+$. These, together with the n.m.r. data given in Table 1, establish its structure as methyl tetrahydroxysterchohanate (methyl $3\alpha,7\alpha,12\alpha,22\xi$ -tetrahydroxy-5 β -cholestan-26-oate, Ib). The corresponding ethyl ester (Ic), prepared by Tammar (1970) showed (except for the ester group) the same n.m.r. spectrum (Table 1) and its Me_3Si derivative exhibited

Table 1. Proton resonances of compounds isolated from *Chelonia mydas* bile
Reference standard, tetramethylsilane; solvent, pentadeuteropyridine. Signals (δ) are singlets unless otherwise indicated.

		δ (p.p.m.)								
Compounds		C-18	C-19	C-21	C-27	3β	7β	12β	22	CO ₂ CH ₃ or CO ₂ C ₂ H ₅
Methyl tetrahydroxystercho- anate (Ib)	0.90	1.03	1.40, d <i>J</i> 6.0 Hz	1.22, d <i>J</i> 7 Hz	$\sim 3.72, \ddagger$ m(br)	4.11, m(n) <i>W</i> $\frac{1}{2}$ 8 Hz	4.35, m(n) <i>W</i> $\frac{1}{2}$ 8 Hz	$\sim 4.00, \dagger$ m(br)	3.63	
Ethyl tetrahydroxystercho- anate (Ic)	0.91	1.04	1.42, d <i>J</i> 6.0 Hz	1.23, $\dagger$ d <i>J</i> 7 Hz	~ 3.74 , m(br)	$\sim 4.1, \dagger$ m(n)	4.35, m(n)	$\sim 4.0, \dagger$ m(br)	4.17, $\dagger$ q 1.16, $\dagger$ t	
Tetrahydroxystercho- lactone (IIa)	0.81	1.03	1.25, $\dagger$ d <i>J</i> 6.5 Hz	1.27, $\dagger$ d <i>J</i> 7 Hz	~ 3.73 , m(br)	4.10, m(n) <i>W</i> $\frac{1}{2}$ 8 Hz	4.25, m(n) <i>W</i> $\frac{1}{2}$ 8 Hz	~ 4.38 , m(br)	—	
Methyl 3 α ,12 α ,22 ξ -trihydroxy- 5 β -cholestan-26-oate (Id)	0.83	0.99	1.38, d <i>J</i> 6.5 Hz	1.22, d <i>J</i> 7 Hz	$\sim 3.90, \ddagger$ m(br)	—	4.30, m(n)	$\sim 4.01, \ddagger$ m(br)	3.65	
3 α ,12 α ,22 ξ -Trihydroxy-5 β - cholestan-26-oic lactone (IIb)§	0.73	0.97	1.22, $\dagger$ d <i>J</i> 6.5 Hz	1.29, d <i>J</i> 7 Hz	*	—	4.20, m(n)	*	—	

* Weak signals not detectable because of insufficient material and/or overlapping signals.

† Signals overlapped by others.

‡ These signals were detected after the addition of $^2\text{H}_2\text{O}$.

§ Impure sample.

a very intense peak in its mass spectrum at m/e 231 (C^+) and small peaks at m/e 383 ($D-3\text{Me}_3\text{SiOH}^+$), 371 ($B-2\text{Me}_3\text{SiOH}^+$), 343 ($A-2\text{Me}_3\text{SiOH}^+$), 293 ($D-4\text{Me}_3\text{SiOH}^+$), 281 ($B-3\text{Me}_3\text{SiOH}^+$), 253 ($A-3\text{Me}_3\text{SiOH}^+$), 185 ($C-C_2\text{H}_5\text{OH}^+$) and 141 ($C-\text{Me}_3\text{SiOH}^+$).

In an analogous way, two other substances, compounds (c) and (d), were obtained from material from zone B (R_F 0.16–0.29) of the t.l.c. separation of bile salts. Compounds (c) and (d) gave i.r. spectra in KBr, which suggested that they both contained the substituted ring nucleus of deoxycholic acid (3 α ,12 α -dihydroxy-5 β -cholan-24-oic acid).

Compound (c), the less polar on t.l.c. in the systems used, gave as its Me_3Si derivative, mass-spectral peaks at m/e 576 (M^+), 471 ($M-\text{Me}_3\text{SiOH}-\text{CH}_3^+$), 396 ($M-2\text{Me}_3\text{SiOH}^+$), 345 ($E-\text{Me}_3\text{SiOH}^+$), 283 ($F-2\text{Me}_3\text{SiOH}^+$) and 255 ($E-2\text{Me}_3\text{SiOH}^+$). The n.m.r. spectrum of compound (c) (Table 1) showed no peak for 7 β -H, indicating the absence of a hydroxy group at C-7 α . These results suggest that compound (c) should be formulated as the lactone (IIb) of 3 α ,12 α -, 22 ξ -trihydroxy-5 β -cholestan-26-oic acid (Ie).

Compound (d), the more polar on t.l.c., gave as its Me_3Si derivative a very intense mass-spectral peak at m/e 217 (C^+) and small peaks at m/e 665 ($M-\text{CH}_3^+$), 385 ($D-2\text{Me}_3\text{SiOH}^+$), 345 ($A-\text{Me}_3\text{SiOH}^+$), 295 ($D-3\text{Me}_3\text{SiOH}^+$), 255 ($A-2\text{Me}_3\text{SiOH}^+$), 185 ($C-\text{CH}_3\text{OH}^+$) and 127 ($C-\text{Me}_3\text{SiOH}^+$). Its n.m.r. spectrum (Table 1) also showed no peak for 7 β -H, but did give a resonance for CH_3 as a methyl ester. It may therefore be formulated as methyl 3 α ,12 α ,22 ξ -trihydroxy-5 β -cholestan-26-oate (Id).

Material from zones C and D of the original preparative-t.l.c. separation of bile salts was evidently heavily contaminated with substances eluted from the silica gel: after hydrolysis and methylation, t.l.c. in system (3) gave spots corresponding to methyl chol-

ate and methyl deoxycholate. G.l.c., as described by Ikawa & Tammar (1976), of the same material gave a large peak with the retention time of the methyl ester of cholic acid and small peaks with retention times of the methyl esters of deoxycholic acid, chenodeoxycholic acid (3 α ,7 α -dihydroxy-5 β -cholan-24-oic acid), hyodeoxycholic acid (3 α ,6 α -dihydroxy-5 β -cholan-24-oic acid) and lithocholic acid (3 α -hydroxy-5 β -cholan-24-oic acid).

After hydrolysis of the total bile salts, the resulting bile acids were methylated and converted into Me_3Si derivatives. G.l.c.–mass spectroscopy of these gave peaks which were attributed to Me_3Si derivatives of compounds (Ib), (Id), (IIa), (IIb), methyl cholate and methyl deoxycholate. Other bile acid derivatives were not detected. Thus the bile salts of *Chelonia mydas* are taurine conjugates of tetrahydroxystercho-
lactone (Ia) and its 7-deoxy derivative (Id), with much smaller amounts of cholic acid, deoxy-
cholic acid and possibly other bile acids. Taurine-
conjugated tetrahydroxystercho-
lactone (Ia) is the principal bile salt.

Discussion

Chemical

Tetrahydroxystercho-
lactone (Ia), its ethyl ester (Ic) and the corresponding lactone (IIa) have previously been characterized chemically (Tammar, 1970, 1974) and the present work confirms their structures by spectroscopy. The methyl ester (Ib) is now described. Its structure can be considered firmly established by its spectral characteristics and by its formation from compound (Ic).

The 7-deoxy methyl ester (Id) and corresponding lactone (IIb) are newly discovered: their structure seems well established by n.m.r. and mass spectro-

scopy. Nevertheless it is desirable that they should be isolated in purified form, undergo elemental analysis and be made by partial synthesis.

Biological

This work presents the first complete analysis of the bile salts of a chelonian. For *Chelonia mydas*, it shows that (1) there is, indeed, very little in common between chelonian bile salts and those of other animal forms and (2) there is probably an enterohepatic circulation of C_{27} bile acids, for, by analogy with C_{24} acids, it is likely that the 7-deoxy acid (Ie) is secondarily formed from tetrahydroxysterchoholic acid or its conjugate by intestinal micro-organisms. Evidence that C_{27} bile acids or their conjugates undergo enterohepatic circulation has not, to our knowledge, previously been obtained. The presence of small amounts of cholic acid and deoxycholic acid conjugates in the bile suggests that, as in almost all other animal forms, evolution from C_{27} to C_{24} bile-salt types is taking place. If this is so, it represents yet another example of the independent evolution of C_{24} bile acids in different groups of vertebrates, a phenomenon that may eventually help in an understanding of the evolutionary process as a whole. In any case the C_{24} acids are unlikely to be dietary artifacts, for this turtle feeds

chiefly or entirely on seaweed. The bile-salt chemistry conforms well with what might be expected from a primitive specialized reptile.

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References

- Amos, B., Anderson, I. G., Haslewood, G. A. D. & Tökés, L. (1977) *Biochem. J.* **161**, 201–204
Anderson, I. G., Haslewood, G. A. D., Oldham, R. S., Amos, B. & Tökés, L. (1974) *Biochem. J.* **141**, 485–494
Haslewood, G. A. D. (1967) *Bile Salts*, p. 26, Methuen, London
Haslewood, E. S. & Haslewood, G. A. D. (1972) *Biochem. J.* **130**, 89 p
Ikawa, S. (1976) *J. Chromatogr.* **117**, 227–231
Ikawa, S. & Tammar, A. R. (1976) *Biochem. J.* **153**, 343–350
Tammar, A. R. (1970) Ph.D. Thesis, University of London
Tammar, A. R. (1974) *Chem. Zool.* **9**, 337–351