Bile acids. XLIX. Allocholic acid, the major bile acid of *Uromastix hardwickii*

S. S. Ali[1], H. Farhat[1], and William H. Elliott[2]

Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri 63104

Abstract Tauroallocholate is the major bile salt of the lizard, *Uromastix hardwickii*. Alkaline hydrolysis of bile from 25 gallbladders provided 1.21 g of acidic material, about 90% of which was allocholic acid. Analyses by gas–liquid chromatography, and mass spectrometry verified the presence of almost 10% of deoxycholic acid and smaller amounts of other 5α- and 5β-bile acids.

Supplementary key words tauroallocholate • lizard bile • allo bile acids • mass spectrometry • gas–liquid chromatography

With the establishment (1) of the structure of allocholic acid, a major component of the bile of the Gigi fish (2) and of the bile of the king penguin (3), the studies of Anderson and Haslewood (1) aroused new interests toward the identification of this acid in other species. As a consequence allocholic acid has been reported as a constituent of the bile of the leopard seal (4), salamander (5), carp (6) and several species of fish, reptiles, birds (7) and several mammals including oxen (6) and man (8). The formation of allocholate and cholate from [4-^{14}C]7α-hydroxycholesterol has been demonstrated in the hen (9), rat, and rabbit (10). 5α-Cholestanol has been shown to be a precursor in the rat (11, 12) and gerbil (13). Pathways of biosynthesis of allocholate from cholestanol or cholesterol have been proposed (12, 14) and several studies suggest that allocholate is a primary bile acid in certain species (6, 9, 10). Unfortunately, very few of these natural sources of allocholate contain appreciable quantities of the acid. This paper describes the isolation and identification of bile acids obtained from gall bladders of *Uromastix hardwickii*, a herbivorous lizard native to Pakistan, in which allocholic acid is the major bile acid, indicating that this species is an excellent source of allocholic acid.

MATERIALS AND METHODS

The animals, 6–12 inches in length found in the Sind Region near Karachi, were obtianed from deep burrows below the sand during their period of hibernation (November to March). The gall bladders from 25 animals were removed, and bile was drained into 95% ethanol to precipitate proteins. After several days the precipitate was removed, and the ethanol extract was evaporated under nitrogen. The dried bile, 2.9 g, was retained at 4°C until hydrolyzed with 200 ml of 2.5 N KOH for 14 hr in an autoclave at 15 lbs psi and 120°C. The alkaline solution was diluted with an equal volume of water, and extracted with ethyl acetate (3 times with 100 ml of solvent). The unsaponified fraction was washed with water, dried over anhydrous sodium sulfate, and evaporated under nitrogen to afford 67.1 mg. The composition of this material is currently under investigation. The aqueous hydrolysate was acidified with 6 N HCl to pH 1 and extracted with ethyl acetate (3 times with 100 ml of solvent); the extract was

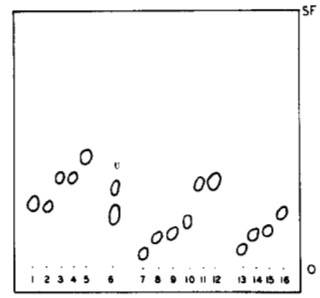

Fig. 1. Thin-layer chromatogram of conjugated bile acids from *Uromastix hardwickii*. Solvent system: 2-propanol–chloroform–ammonium hydroxide 30:16:1 (v/v); double development. The numbers refer to the following compounds: 1, taurocholate; 2, tauroallocholate; 3, tauroallodeoxycholate; 4, tauroallochenodeoxycholate; 5, taurolithocholate; 6, *Uromastix* bile salts; 7, glycoallocholate; 8, glycoallodeoxycholate; 9, glycoallochenodeoxycholate; 10, glycoallolithocholate; 11, taurodeoxycholate; 12, taurochenodeoxycholate; 13, glycocholate; 14, glycodeoxycholate; 15, glycochenodeoxycholate; 16, glycolithocholate. SF = solvent front; 0 = origin.

Abbreviations: TLC, thin-layer chromatography; GLC, gas-liquid chromatography; TMSi, trimethylsilyl; RRT, relative retention time.

Systematic nomenclature of the compounds referred to in the text by their trivial names is as follows: allocholic acid, 3α,7α,12α-trihydroxy-5α-cholan-24-oic acid; allochenodeoxycholic acid, 3α,7α-dihydroxy-5α-cholan-24-oic acid; allodeoxycholic acid, 3α,12α-dihydroxy-5α-cholan-24-oic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholan-24-oic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholan-24-oic acid; cholic acid, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid; cholestanol, 5α-cholestan-3β-ol.

[1] Department of Biochemistry, University of Karachi, Karachi 32, Pakistan.

[2] Author to whom to address correspondence.

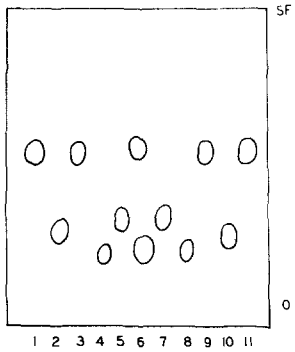

Fig. 2. Thin-layer chromatogram of free bile acids after alkaline hydrolysis. Solvent system: chloroform–methanol–acetic acid 80:12:13 (v/v). The numbers refer to the following compounds: 1 and 11, chenodeoxycholate; 2 and 10, cholate; 3 and 9, allochenodeoxycholate; 4 and 8, allocholate; 5 and 7, 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid; 6, *Uromastix* free bile acids. SF = solvent front; E = origin.

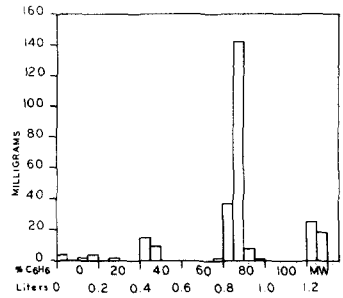

Fig. 3. Partition chromatography of 200 mg of free bile acids from *Uromastix*. Fractions 40-1 and 40-2 contained 24.6 mg and fraction 80-1, 80-2 and 80-3 contained 187.1 mg. The mass in the methanol wash (MW) was primarily column packing (Celite).

washed with water and evaporated under nitrogen to provide 1.21 g of acidic material.

Bile acids were separated by acetic acid partition chromatography (15). The fractions are designated according to the percentage of benzene in hexane; e.g., fraction 80-2 represents the second fraction of eluent containing 80% benzene in hexane. Analytical TLC and GLC were carried out as described previously (13). Mass spectra were determined with an LKB 9000 gas chromatograph–mass spectrometer (LKB Instruments, Rockville, Md.) with a column of 1% OV-17 (column, 260°C; ion source, 270°C) or via the direct probe (ionizing energy, 70eV, accelerating voltage, 3.5 Kv). Melting points and infrared spectra in potassium bromide pellets were determined as reported (13). Proton magnetic resonance spectra were taken with a Varian A-60 spectrometer (Varian Assoc., Palo Alto, Cal.) at 60 MHz and tetramethylsilane as internal standard (16). For comparative purposes allo bile acids were prepared from appropriate methyl cholanates (12).

RESULTS

Fig. 1 shows a thin-layer chromatogram of the conjugated bile acids from *Uromastix hardwickii* compared with several known derivatives. The major material contained predominately taurotrihydroxy acid (6) with lesser amounts of taurodihydroxy derivatives. After alkaline hydrolysis (**Fig. 2**) large spots appeared in the areas related to allocholic acid and dihydroxy acids, with very minor spots occurring just above these two areas. This distribution was confirmed by acetic acid partition chromatography (**Fig. 3**) of 200 mg of the acidic material. The major amount of product (187.1 mg) was obtained in fractions 80-1 through 80-3. After crystallization from methanol–acetone, the product exhibited mp 238–243°C [reported mp 238–240°C (11)]; infrared spectra showed prominent bands at 9.2, 9.7, 9.9, 10.45, and 11.25 μ. The double peak observed at 9.7 and 9.9, and the band at 11.25 μ have been described as characteristic of allocholic acid (7). The acid was methylated with diazomethane and crystallized from methanol–acetone, mp 226–228°C [reported mp 225–226°C (1)]. The proton magnetic resonance spectrum showed the following chemical shifts: $C_{12}H$, 6.04(m); C_7H, 6.12(m); C_3H, 6.23(m); $C_{19}H$, 9.24(s); $C_{18}H$, 9.32(s); methyl ester, 6.36(s). A sample of authentic methyl allocholate gave the following: $C_{12}H$, 6.04(m); C_7H, 6.11(m); C_3H, 6.23(m); $C_{19}H$, 9.24(s); $C_{18}H$, 9.31(s); methyl ester, 6.37(s). The mass spectrum showed a very weak molecular ion, *m/e* 422, and characteristic fragments at *m/e* 404, [M-18]; 386, [M-(2 × 18)]; 368, [M-(3 × 18)]; 353, [M-(3 × 18 + 15)]; 289 [M-(18 + side chain)]; 271, [M-(2 × 18 + side chain)] (base peak); and 253, [M-(3 × 18 + side chain)].

Material in fractions 40-1 and 40-2 (24.6 mg) was crystallized from a mixture of hexane and acetone to provide a product; mp 177°C [reported for deoxycholic acid, mp 177°C (17)]. A sample of the acid was methylated with diazomethane, converted to the trimethylsilyl ether and examined by mass spectrometry. The mass spectrum exhibited a weak molecular ion, *m/e* 550, and characteristic fragment ions of *m/e* 535, [M-15], 460, [M-90]; 370, [M-(2 × 90)]; 345, [M-(90 + 115)]; and 255, [M-(2 × 90 + 115)] base peak, characteristic of a 12-hydroxylated bile acid (18).

Quantitation of the bile acids by gas–liquid chromatography of the methyl esters and their TMSi ethers on 3% QF-1 and 3% OV-17 (**Table 1**) showed the presence of minor amounts of chenodeoxycholate, allodeoxycholate, allochenodeoxycholate and cholate, as well as two unidentified acids constituting 0.38 and 0.85% of the total bile acid composition.

DISCUSSION

Abundant natural sources of allo bile acids are of particular interest to those concerned with a study of the physiological importance of these acids. To date small, more primitive animals appear to be better sources of these acids, but few quantitative data are available. Anderson and Haslewood (19) have reported allocholic acid as a constituent of the North American white sucker *Catostomus commersoni lacepede*, although 5α-chimaerol sulfate is the dominant bile salt. In bile of the river carpsucker *Carpiodes carpio* (Rufenesque) (Catostomidae) tauroallocholate is the major bile salt (20).

TABLE 1. Composition and relative retention times of bile acids of gall bladder bile in *Uromastix Hardwickii*

Bile Acid	% Total	Me Esters				TMS Me Ester			
		QF-1[a]		OV-17[a]		QF-1		OV-17	
		standard	*sample*	*standard*	*sample*	*standard*	*sample*	*standard*	*sample*
Unknown	0.38		0.70		0.77		0.64		0.60
Deoxycholic	9.65	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Chenodeoxycholic	0.38	1.18	1.17	1.14	1.14	1.09	1.09	1.00	1.00
Allodeoxycholic	0.59	1.03	1.03	1.19	1.19	0.93	0.91	1.22	1.22
Allochenodeoxycholic	0.45	1.22	1.22	1.27	1.26	1.00	1.00	0.92	0.92
Unknown	0.85		1.61		1.66		1.70		1.73
Cholic	0.50	2.33	2.39	2.19	2.18	1.05	1.05	0.90	0.91
Allocholic	87.21	2.68	2.68	2.51	2.52	1.00	1.00	0.87	0.85

[a] 3% silicones on Gas-Chrom Q as phases for gas chromatography.

5α-Cyprinol (21), and allochenodeoxycholic acid (22) have been isolated from carp bile, but 5α-cyprinol sulfate is the major bile salt. From the bile of 53 gall bladders from carp 10 mg of crystalline methyl allocholate was obtained (6). Tammar (23) has recently reviewed the nature of bile salts in fishes.

The large percentage of allocholic acid (about 90%) present as the tauro derivative in bile of the lizard, *Uromastix hardwickii*, emphasizes the importance of this species as a source of allo acid. *Uromastix microlepis*, native to Kuwait, contains a similar high content of allocholate[3], and the tauro derivative has also been detected chromatographically in bile from *Uromastix thomasi* (7): The major bile acid from the lizard *Iguana iguana* (24) is reported to be allocholic acid. These observations indicate the presence of an active 12α-hydroxylase in the biosynthesis of bile acids in these lizards.

If the processes of enterohepatic circulation and bacterial 7α-dehydroxylation in the intestine occur in lizards as in larger animals, the presence of allodeoxycholic acid in bile of *Uromastix hardwickii* as a secondary derivative from allocholic acid would be anticipated, as would the presence of deoxycholate from cholate. However, the ratio of allocholate to allodeoxycholate (148:1) is drastically shifted in comparison to the ratio of cholate to deoxycholate (0.05:1). Whether this marked difference is due to radical behavior by a few animals, related to diet, time of collection during hibernation, or other factors must await further experiments to delineate metabolic pathways of biosynthesis of these bile acids in this species.

These studies were supported in part by U. S. Public Health Service Grant HL-07878 and by the University of Karachi.

The capable technical assistance of Eric Stephenson and William Frasure is gratefully acknowledged. Dr. Roger Shaw kindly provided samples of conjugated bile acids.

Manuscript received 17 July 1975; accepted 17 September 1975

REFERENCES

1. Anderson, I. G., and G. A. D. Haslewood. 1962. Comparative studies of bile salts. 15. The natural occurrence and preparation of allocholic acid. *Biochem. J.* **85:** 236–242.
2. Haslewood, G. A. D., and I. D. P. Wootton. 1956. The chemical nature of tetrahydroxynorsterocholanic acid. *Biochem. J.* **63:** 3p.
3. Anderson, I. G., G. A. D. Haslewood, and I. D. P. Wootton. 1957. Comparative studies of bile salts. 10. Bile salts of the king penguin, *Aptenodites patagonica*. *Biochem. J.* **67:** 323–328.
4. Haslewood, G. A. D. 1961. Comparative studies of bile salts. 13. Bile acids of the leopard seal, *Hydrurga leptonyx*, and of two snakes of the genus *Bitis*. *Biochem. J.* **78:** 352–359.
5. Amimoto, K. 1966. Stereo-bile acids and bile alcohols. LXXVIII. Bile salts of the salamander, *Megalobatrachus japonicus*. *J. Biochem.* (*Tokyo*). **59:** 340–343.
6. Yamasaki, K., S. Ikawa, Y. Ayaki, and Y. Yamamoto. 1972. Natural occurrence of allocholic acid in ox and carp bile. *J. Biochem.* (*Tokyo*). **72:** 769–772.
7. Haslewood, G. A. D. 1967. Bile Salts. Methuen, London, 82–106.
8. Eneroth, P., B. Gordon and J. Sjovall. 1966. Characterization of trisubstituted cholanoic acids in human feces. *J. Lipid Res.* **7:** 524–530.
9. Yamasaki, H., and K. Yamasaki. 1972. In vivo conversion of 7α-hydroxycholesterol-^{14}C to 3α,7α-dihydroxychol-5-enoic-^{14}C and 4-enoic-^{14}C acids as well as to allocholic-^{14}C acid in the hen. *J. Biochem.* (*Tokyo*). **71:** 77–83.
10. Yamasaki, K., Y. Ayaki, and G. Yamasaki. 1972. Allocholic acid, a metabolite of 7α-hydroxycholesterol in the rat and rabbit. *J. Biochem.* (*Tokyo*). **71:** 927–930.
11. Karavolas, H. J., W. H. Elliott, S. L. Hsia, E. A. Doisy, Jr., J. T. Matschiner, S. A. Thayer and E. A. Doisy. 1965. Bile acids. XXII. Allocholic acid, a metabolite of 5α-cholestan-3β-ol in the rat. *J. Biol. Chem.* **240:** 1568–1572.
12. Elliott, W. H. 1971. *In* The Bile Acids, P. P. Nair and D. Kritchevsky, editors, Plenum, New York, 47–93.
13. Noll, B. W., L. B. Walsh, E. A. Doisy, Jr. and W. H. Elliott. 1972. Bile acids, XXXV. Metabolism of 5α-cholestan-3β-ol in the Mongolian gerbil. *J. Lipid Res.* **13:** 71–77.
14. Björkhem, I., and J. Gustafsson. 1971. On the conversion of cholestanol into allocholic acid in rat liver. *Eur. J. Biochem.* **18:** 207–213.
15. Matschiner, J. T., T. A. Mahowald, W. H. Elliott, E. A. Doisy, Jr., S. L. Hsia, E. A. Doisy. 1957. Bile acids. I. Two new acids from rat bile. *J. Biol. Chem.* **225:** 771–779.
16. Shalon, Y., and W. H. Elliott. 1974. Identification of bile acids as their derivatives by 100 MHz PMR spectroscopy. *FEBS Lett.* **41:** 223–226.

[3] Haslewood, G. A. D., personal communication.

17. Fieser, L. F., and M. Fieser. 1959. Steroids, Reinhold, New York, 54.
18. Elliott, W. H. 1971. *In* Biochemical Application of Mass Spectrometry, G. R. Waller, editor. Wiley, New York, 291–312.
19. Anderson, I. G., and G. A. D. Haslewood. 1970. Comparative studies on bile salts. 5α-chimaerol, a new bile alcohol from the white sucker *Catostomus hommersoni lacepede*. *Biochem. J.* **116:** 581–587.
20. Briggs, T., and C. Bussjaeger. 1972. Allocholic acid, the major component in bile from the river carpsucker, *Carpiodes carpio*. *Comp. Biochem. Physiol.* **42B:** 493–496.
21. Hoshita, T. 1962. Stero-bile acids and bile sterols. XLVI. Isolation of a new bile sterol, 3α,7α,12α-trihydroxy-26,27-epoxycholestane from carp bile. *J. Biochem.* (*Tokyo*). **52:** 125–130.
22. Yamaga, N. 1971. Isolation of allochenodeoxycholic acid from bile of carp (*Cyprinus carpio*). *Yonago Acta Med.* **15:** 18–20.
23. Tammar, A. R. 1974. Bile Salts in Fishes *In* Chemical Zoology, Academic Press, New York, Vol. VIII, 595–612.
24. Okuda, K., M. G. Horning and E. C. Horning. 1972. Isolation of a new bile acid, 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid, from lizard bile. *J. Biochem.* (*Tokyo*). **71:** 885–890.