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STUDIES ON THE BILE ACIDS OF THE GALL BLADDER BILE OF A MUD-*PUPPY, SALAMANDER NECTURUS.*

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ABSTRACT

The major bile acids of gall bladder bile of salamander necturus have been reported and shown to be taurine conjugates. The bile acid conjugate were hydrolysed and the free bile acids were separated by thin layer chromatography and identified through gas liquid chromatography and mass spectrometry (GC-MS) as methyl esters and methyl-ester trimethyl silyl ethers. About 56.3% of the total bils arid was present as allocholic add whereas 3α, 7α, 12α -dihydroxy-5α -cholestanoic acid and cholic acid represented 30.4 and 12.3% respectively. Dihydroxy bile acids such as deoxycholic allodexycholic and allochenodexycholic were present in less than 0.5% of the total bile acids.

Introduction

The bile acids which occur in the bile of mammals, birds, some snakes and bony fishes are normally the hydroxy derivatives of cholanic acids. Several species among fishes (Tammer, 1974a ; Haslewood, 1978) amphibians (Haslewood, 1978; Hoshita, 1968 and Tammer 1974b); reptiles (Haslewood, 1978 and Tammer, I974c) and birds (Haslewood, 1967) are known to contain the allocholanic nucleus. In some primitive animals bile acids and bile alcohols containing a complete side chain of cholesterol molecule have been isolated and identified (Haslewood, 1967). The wide distribution of C_{27} bile acids and bile alcohols having both normal and allo configuration at the five position initiated the expanded interest in the investigations in this area. Such studies yielded new structures in the bile acid and bile alcohol nomenclature and provided an opportunity to establish alternative biogenetic pathways that were involved in the formation of such compounds from its precursor, cholesterol. Among amphibians, *Urodeles,* Salamanders, and *Anurans* (frogs and toads) were most attractive because of their metamorphotic ability. The Urodeles examined so far are the Congo eel, *Amphiuma means,* (Kihara *et al.,* 1967); Salamander *Diemyc*t*ylus pyrrhogast*er, (Kihara *et al*., 1977); giant Salamander, *Megalobatrachus Japonicas,* (Amimoto, 1966) and Fire Salamander, *Salamander salamandra,* (Tammar, 1974c). A preliminary report on a mud-puppy, *Salamander necturus* has been presented (Ali, 1975). The present paper describes the composition of bile acids obtained from the gallbladder bile of *Salamander necturus.*

Materials and Methods

Reference Compounds:

Samples of deoxycholic, cholic, chenodeoxycholic, allocholic, allocheno-deoxyecholic were available from commercial sources. 3α, 7α, 12α-trihydroxy-5β-cholestanoic acid was a gift from Dr. Russel. F. Hanson, University of Minnesota Medical School, Minneapolis, M.N. 3α, 7α, 12α -trihydroxy-5α-cholestanoic acid was derived from carp bile, (Kamat and Elliott, 1972).

Extraction and isolation of bile acids.

Bile of *Salamander necturus* (3.7 ml) was a gift from Dr. Nicholas, Department of Biochemistry, St. Louis University School of Medicine, St. Louis, MO. Ethyl alcohol was added to the bile and left for several days. Proteins were removed by filtration and the solvent was evaporated by a slow stream of nitrogen to provide 255 mg of dried bile salts. The dried material was hydrolyzed with 25 ml of 2.5 N KOH in an autoclave at 121°C for 14 hours. A portion of the dried salt was subjected to thin layer chromatography for their conjugates. The hydrolyzed bile was diluted with an equal volume of water and the unsaponifiable matter was extracted from the alkaline hydrolyzed three times with ethyl acetate. The solvent layer was thoroughly dried with anhydrous sodium sulfate and evaporated to dryness. The alkaline phase of the hydrolyzed material was acidified with 6 N-HCl to pH 1 and extracted with ethyl acetate (3 times with 50 ml solvent). After washing the solvent layer with water, drying the extract with anhydrous sodium sulfate and evaporation under nitrogen, yielded 63 mg of bile acids for further analysis.

Chromatographic analysis and GC-mass spectrometry.

Conjugated bile salts were separated on a thin layer silica gel G plate in a solvent system: 2-propyl alcohol: Chloroform: ammonium hydroxide, 30:16:1 (v/v) along with standards (Ali *et al,* 1976). Free bile acids were chromatographed on a silica gel G plate using chloroform: methanol: acetic acid 85:12:3 (v*/v)* with known standards (Amimoto, 1966b). Analytical gas liquid chromatography of bile acid was performed on 3% OV-17 and QF-1 columns as methyl esters and their methyl ester trim ethyl silyl ether derivatives. The gas chromatography-mass spectrometry (GC-MS) of methyl esters and trim ethyl silyl ethers of bile acids was carried out using OV-1 column with a LKB 9000 gas chromatograph-mass spectrometer (LKB instrument, Rockville, MD) with an ionizing energy, 70eV and accelerating voltage ranging from 2.1-3.5 Kv.

Results and Discussion

Thin layer chromatography of the dried bile prior to hydrolysis shows that the bile acids in the bile of *Salamander necturus* were existed as their taurine conjugates (Figure 1) and after alkaline hydrolysis indicated a mixture of several constituents of bile acids on thin layer chromatography (Figure 2). On comparison with authentic standards the presence of allocholic, 3α, 7α, 12α-trihydroxy-5α-cholastanoic, and cholic acids along with other compounds were indicated. The individual bile acids were further identified on the basis of their retention data by GLC with respect to their methyl esters and trim ethyl silyl ethers of deoxycholic acid as well as comparision with GC-MS.

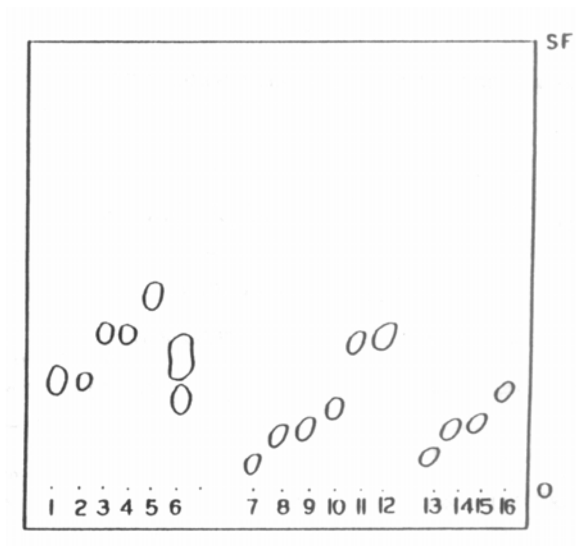

Figure-1. Thin layer chromatogram of conjugated bile acids from *Salamander necturus.* Solvent System: 2-propylalcohol-chloroformammonium hydroxide 30:16:1 (V/V) double development. The numbers refer to the following compounds: **1**. taurocholate **2**. tauroallochlate **3**. tauroallodeoxycholate **4**. tauroallochenodeoxycholate **5**. taurolithocholate **6.** *Salamander necturus* bile salts **7**. glycoallocholate **8**. Glycoallodeoxycholate; **9**. Glycoallochenodeoxycholate, **10**. Glycolithocholate **11**. Taurodeoxycholate **12**. Taurochenodeoxycholate **13**. Glycocholate **14**. Glycodeoxycholate **15**. Glycochenodeoxycholate **16**. Glycolitho-cholate. SF = Solvent front; O = origin.

TABLE-1

Composition and relative retention times of bile acids from *Salmander Necturus*.

Bile Acid	%total	Methyl Ester				TMS Methyl Ester			
		QF-1		OV-17*		QF-1		V-17	
		Standard	Sample	Standard	Sample	Standard	Sample	Standard	Sample.
Deoxycholic	0.5	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Allodeoxycholic	0.3	1.07	1.06	1.19	1.17	0.93	0.92	1.22	-
Allochenodeoxycholic	0.2	1.22	1.24	1.27	1.25	1.00	1.00	0.92	0.91
Cholic	12.3	2.37	2.38	2.07	2.08	1.05	1.06	0.90	0.81
Allocholic	56.3	2.68	2.71	2.51	2.50	1.00	1.00	0.81	0.81
3α,7α,12α-trihydroxy-5α-cholestanoic	30.4	4.24	4.24	4.18	4.14	1.49	1.47	1.30	1.29

* 3% silicones on Gas-Chrom Q as phases for gas chromatography.

TABLE-2

Fragment ions and their relative intensities of bile acids methyl esters of the gall bladder bile of *Salmander Necturus*

Fragments	DIHYDROXY				TRIHYDROXY					
	1		2		3		4		5	
	ions	%	ions	%	ions	%	ions	%	ions	%
[M]+	406	11.4	406	-	422	tr	422	tr	464	0.5
[M-18]+	388	65.1	388	4.7	404	5.2	404	5.2	446	2.9
[M-18+15]+	373	20.3	373	8.5	-	-	-	-	-	-
[M-36]+	370	24.0	370	12.8	386	35.6	386	33.8	428	31.7
[M-36]+	355	7.0	355	2.6	371	6.3	371	6.5	413	6.0
[M-54]+	-	-	-	-	368	6.8	368	28.6	410	4.3
[M-54+15]+	-	-	-	-	353	5.4	353	14.3	315	16.2
[M-18+Side Chain]+	273	100.0	273	81.5	289	9.4	289	11.8	289	11.9
[M-36+Side Chain]+	255	38.0	255	100.0	271	100.0	271	87.0	271	100.0
[M-54+Side Chain]+	-	-	-		253	26.5	253	100.0	253	21.4
	288	46.8	228	8.5	261	46.9	261	32.5	303	35.7
	213	69.6	213	9.4	226	10.0	226	28.6	247	9.0
	-	-	-	-	211	11.7	211	28.6	229	7.2

Methyl esters of 1.Allodeoxycholic 2.Deoxycholic 3.Allocholic 4.Cholic 5.3α, 7α, 12α-trihydroxy-5α- cholestanoic acid.

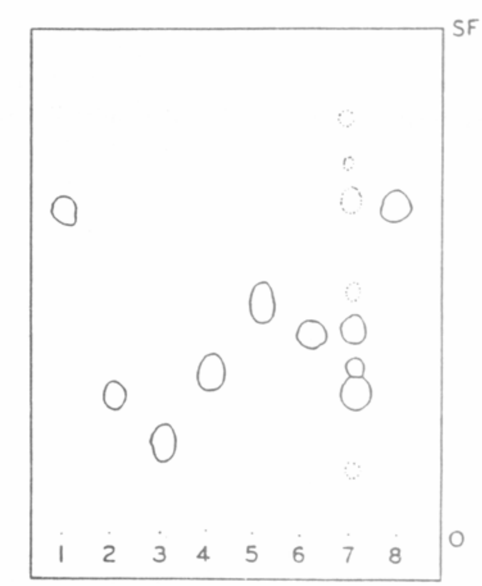

Figure-2. Thin layer chromatogram of free bile acids of *Salamander necturus* Solvent System: chloroform; methanol: acetic acid 85:12:3 (v/v). The number refers to the following compounds: 1 and 8. allochcnodcoxycholic 2. allocholic 3. varanic 4. cholic 5. 3α, 7α, 12α-trihyhydroxy-5β-cholestanoic 6. 3α, 7α, 12α-trihydroxy-5α-cholestanoic 7. *Salamander necturus* free bile acids.

Table 1 gives the relative amounts and the Gas chromatographic retention data of bile acids isolated from *Salamander necturus*. Table 2 gives the mass spectrometric data of the bile acid mature as their methyl esters. A combination of TLC (Figure 2), GLC (Table 1) and GC-MS (Table 2) confirmed the presence of allocholic (56%), 3α, 7α, 12α-trihydroxy-5α -cholestanoic (30.4%) and Cholic 12%) as major constituents. Among other acids, allodeoxycholic, deoxycholic and allochenodeoxycholic acid were found to occur in the bile in detectable quantities. The bile methyl esters were also converted into their trimethyl silyl ethers and subjected to GC-MS. Peaks I and II (Figure 3) compare with the mass spectrum of standard allocholic and cholic acid derivatives. Both allocholic and choke acids derivatives provided ions at m/z 638 $[M]^+$; 623 $[M\text{-}15]^+$; 548 [M-90]; 533 $[M\text{-}90+ 15]^+$; 458 $[M\text{-}180]^+$; 443 $[M\text{-}180 + 15]^+$; 368, $[M\text{-}270]^+$; 353 $[M\text{-}270+15]^+$; 343 $[M\text{-}180\text{- side chain}]^+$; and 253 $[M\text{-}270\text{+ side chain}]^+$. The abundance of the fragment m/z 261 was greater in allocholate (73.6%) than cholate (7.2%). Similarly allocholate contained intense ions at m/z 458 and 343 as compared to cholate. Peak IV was found to be 3α, 7α, 12α -trihydroxy-5α-cholestanoic acid. The predominant ions were seen at m/z 680 $[M]^+$ 10.9%; 665 $[M\text{-}15]^+$ 97.7%; 590, $[M\text{-}90]^+$ 83.1%; 575 $[M\text{-}90+15]^+$ 39.4%, 500

$[M-180]^+$ 100%, base peak; 485 $[M-180+15]^+$ 6.4%; 410, $[M-270]^+$ 9.9%; 395 [M-270 + $15]^+$ chain]. Fragments of nuclear cleavages at m/z 226 (2.9%) and 211 (6.4%) were also encountered. Peak V was found to be a pentahydroxy alcohol which might be a contaminant ion with the acidic fraction during extraction.

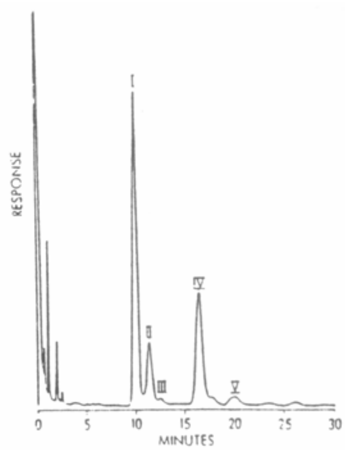

Figure-3. Gas liquid chromatogram of bile acid methyl esters of *Salamander necturus* as their trimethyl silyl ether derivatives on 3% OV-17 at 260°C. The identities of peaks are provided in the text.

The major bile acids in the bile of *Salamander necturus* were allocholic and 3α, 7α, 12α-trihydroxy-5α-cholestanoic acids and compare in composition with a lizard, iguana iguana, Haslewood (1967). Allocholic acid is widely distributed in lizards, (Ali *et al*, 1976 and Ali, 1979) mostly as tauroconjugates. The giant salamander, Megaolbatrachus Japonicus contained allocholic acid, (Amimoto, 1966a) but 3α, 7α, 12α -trihydroxy-5α-cholestanoic acid was not detected. However, acidic metabolites were detected after intraperitoneal administration of tritium labelled 27-deoxy-5α-cyprinol, (Amimoto, 1966c). Small activity was found to be associated with allocholic acid and another unidentified tetrahydroxy-5α-cholestanic acid (Amimoto, 1966c). Salamander necturus has been found to be a good source of providing allo compounds especially allocholic and 3α, 7α, 12α-trihydroxy-5α-cholestanoic acid. It appears that there is some biochemical link between Salamander and Lizards.

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