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Bile Acids of Snakes of the Subfamily Viperinae and the Biosynthesis of C-23-Hydroxylated Bile Acids in Liver Homogenate Fractions from the Adder, *Vipera berus* (Linn.)

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1. Analysis of bile salts of four snakes of the subfamily Viperinae showed that their bile acids consisted mainly of C-23-hydroxylated bile acids. 2. Incubations of ^{14}C-labelled sodium cholate (3α,7α,12α-trihydroxy-5β-cholan-24-oate) and deoxycholate (3α,12α-dihydroxy-5β-cholan-24-oate) with whole and fractionated adder liver homogenates were carried out in the presence of molecular oxygen and NADPH or an NADPH-generating system. The formation of C-23-hydroxylated bile acids, namely bitocholic acid (3α,12α,23ξ-trihydroxy-5β-cholan-24-oic acid) and 3α,7α,12α,23ξ-tetrahydroxycholanic acid (3α,7α,12α,23ξ-tetrahydroxy-5β-cholan-24-oic acid), was observed mainly in the microsomal fraction and partly in the mitochondrial fraction. 3. Biosynthetic pathways of C-23-hydroxylated bile acids are discussed.

The many snakes whose bile salts have been examined fall into three groups, having cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid), pythocholic acid (3α,12α,16α-trihydroxy-5β-cholan-24-oic acid) or C-23-hydroxylated 5β-cholan-24-oic acids as their principal bile acids (Haslewood, 1967*a*). All these acids occur as taurine conjugates.

C-23-hydroxylated bile acids, first isolated from two snakes of the genus *Bitis* (Haslewood, 1961), are not found in all snakes, but appear to be confined to species of Viperinae and Colubridae (Tammar, 1974). The two C-23-hydroxylated bile acids known from snakes are bitocholic acid (3α,12α,23ξ-trihydroxy-5β-cholan-24-oic acid) and 3α,7α,12α,23ξ-tetrahydroxycholanic acid (3α,7α,12α,23ξ-tetrahydroxy-5β-cholan-24-oic acid).

Pythocholic acid is formed in python liver by 16α-hydroxylation of deoxycholic acid which in its turn results from 7α-dehydroxylation of cholic acid by intestinal micro-organisms (Bergström *et al.*, 1960). Pythocholic acid is thus a secondary bile acid, a response, peculiar to boid snakes, to the arrival of deoxycholic acid in the liver via the enterohepatic circulation.

We wished to test further the suggestion that C-23-hydroxylated bile acids are characteristic of the subfamily Viperinae, and also to determine whether adder (*Vipera berus*) livers could effect a direct hydroxylation at C-23, or whether such a hydroxylation is an artifact of the enterohepatic circulation.

Experimental

Materials and methods

Sodium [24-^{14}C]cholate (sp. radioactivity 53 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and [24-^{14}C]-deoxycholate (sp. radioactivity 47.3 mCi/mmol) from International Chemical and Nuclear Corp., Cleveland, Ohio, U.S.A. The purity of these compounds was checked by t.l.c. and radioautography.

The following were kindly provided from Professor G. A. D. Haslewood's collection: bile salts from *Bitis arietans*, *Bitis nasicornis* and *Vipera russelli*, cholic acid, allocholic acid (3α,7α,12α-trihydroxy-5α-cholan-24-oic acid), bitocholic acid, phocaecholic acid (3α,7α,23ξ-trihydroxy-5β-cholan-24-oic acid), 3α,12α-dihydroxy-7-oxo-5β-cholan-24-oic acid, 3α,7α-dihydroxy-12-oxo-5β-cholan-24-oic acid, 3α,7β,12α-trihydroxy-5β-cholan-24-oic acid and 3α,7α,12α,23ξ-tetrahydroxycholanic acid (the last two acids originally from Professor S. Bergström).

Bile salts from *V. berus* were prepared as described by Haslewood (1967*b*) from the gall-bladder bile of the adders used in the experiments. Deoxycholic acid was obtained from BDH Chemicals, Poole, Dorset, U.K.

12α-Hydroxy-3-oxo-5β-cholan-24-oic acid was prepared from methyl deoxycholate as described by Jones *et al.* (1949). The product had m.p. 151°C; reported m.p. 156–158°C.

7α,12α-Dihydroxy-3-oxo-5β-cholan-24-oic acid was prepared from cholic acid as described by Haslewood (1944). The product had m.p. 179–180°C; reported m.p. 179–180°C.

7α,12α-Dihydroxy-3-oxo-4-cholen-24-oic acid was prepared by the method of Kallner (1967). The residue from SeO_2 oxidation of 2g of methyl 7α,12α-dihydroxy-3-oxo-5β-cholan-24-oate was purified on a column of 50g of silicic acid (100 mesh, Mallinckrodt Chemical Works, St. Louis, Mo., U.S.A.). Eluents of chloroform with progressively increasing concentrations of ethanol were used. The required compound was eluted with chloroform/ethanol (19:1, v/v). Recrystallization from aq. methanol gave needles, m.p. 227–228°C, $[\alpha]_D^{20}+56\pm2°$ (*c* 0.38 in methanol), $\lambda_{max.}$ 244nm, ε 14500; reported m.p. 231–233°C, $[\alpha]_D^{20}=+61°$, $\lambda_{max.}$ 244nm, ε 14600.

3β,7α,12α-Trihydroxy-5β-cholan-24-oic acid was prepared as described by Danielsson *et al.* (1962). The product had m.p. 200–201°C; reported m.p. 200–202°C.

Sodium taurocholate and sodium taurodeoxycholate were prepared as described by Norman (1955).

Kieselgel G and H (type 60) were purchased from E. Merck, Darmstadt, Germany, silica-gel sintered plates were from Reeve Angel Scientific, London S.E.1, U.K., and pyrene from Hopkin and Williams, Chadwell Heath, Essex, U.K. NADPH, D-glucose 6-phosphate, isocitric acid and isocitrate dehydrogenase (EC 1.1.1.42) were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.

Solvents (except analytical-quality acetic acid) for chromatography were redistilled at 4–6 weekly intervals; methanol and ethanol were redistilled over KOH.

Adders were obtained from J. G. Animals, 19 Streatham Vale, London S.W.16, U.K.

Analysis of bile salts

Methods for preparation of bile acids and their methyl esters were as described by Haslewood (1967*b*).

T.l.c. and densitometry

Methyl esters of bile acids were subjected to t.l.c. on glass plates (20cm×20cm coated with a 0.25mm-thick layer of Kieselgel G or H), developed with solvent system 1 [benzene/propan-2-ol/acetic acid (15:5:1, by vol.)]. They were revealed by spraying with Usui's (1963) reagent, and the resulting chromatograms were analysed with a Chromoscan MK II integrating densitometer (Joyce, Loebl and Co., Gateshead, Co. Durham, U.K.). Quantitative measurement was carried out by using calibration curves of standard samples. Rechromatography of the eluates from zones marked with I_2 vapour was carried out by using solvents described by Eneroth (1963).

I.r. spectroscopy

This was as described by Anderson *et al.* (1974).

G.l.c.

Methyl esters of bile acids or their trimethylsilyl ethers were chromatographed in a series 104 (model 24) dual-flame-ionization chromatograph (W. G. Pye and Co., Cambridge, Cambs., U.K.), with a coiled glass column (length 152cm, internal diameter 3.5mm) packed with Gas-Chrom Q impregnated with 3% Polysulfone (Applied Science Laboratories, State College, Pa., U.S.A.). The column temperature was 315°C. Retention times of methyl esters relative to methyl deoxycholate (1.00) were: lithocholate, 0.43; chenodeoxycholate, 1.14; bitocholate, 1.32; cholate, 2.64; 3α,7β,12α-trihydroxy-5β-cholan-24-oate, 2.85; allocholate, 2.96. Trimethylsilyl ethers of methylated bile acids were analysed as described by Anderson *et al.* (1970). Retention times of methyl trimethylsilyl ethers relative to methyl trimethylsilyl cholate were: allocholate, 0.87; bitocholate, 1.33; 3α,7α,12α,23ξ-tetrahydroxycholanate, 1.19.

Alumina column chromatography

Bile acid methyl esters were separated on columns of alumina, neutralized as described by Evans & Shoppee (1953), by using eluents of ethyl acetate with progressively increasing concentrations of methanol. Methyl esters of dihydroxy, trihydroxy and tetrahydroxy bile acids were mainly eluted with ethyl acetate, ethyl acetate/methanol (19:1, v/v) and ethyl acetate/methanol (9:1, v/v) respectively. Eluates of each fraction were crystallized as described by Haslewood & Wootton (1950). No crystallized eluate was subjected to preparative t.l.c.

Subcellular fractionation of adder liver

Two sets of two specimens of *V. berus* each weighing about 150g, were kept for 1 week, during which they did not eat. They were killed in a guillotine after spending 1 night in a room at 3–4°C. The livers were removed and homogenized (1g of tissue in 10ml of homogenizing medium) in a Potter–Elvehjem homogenizer with a loosely fitting Teflon pestle. The homogenizing medium was K_2HPO_4 (0.1M) adjusted to pH7.4 with HCl (10M), to which sucrose was added to give a concentration of 0.125M. The homogenates were centrifuged at 800*g* for 10min. The mitochondrial fraction was prepared by centrifugation of the 800*g* supernatant at 10000*g* for 15min. The precipitate was resuspended in the homogenizing medium

and re-centrifuged at 10000*g* for 15min. The microsomal fraction was prepared by centrifugation of the 10000*g* supernatant at 100000*g* for 60min. The precipitate was rinsed with and suspended in the homogenizing medium.

The contamination of the mitochondrial fraction with microsomal fraction and vice versa was estimated from determination of glucose 6-phosphatase (EC 3.1.3.9) (Wilgram & Kennedy, 1963) and succinate–cytochrome *c* reductase (EC 1.3.99.1) activities (Kuff & Schneider, 1954). The mitochondria were thus judged to be contaminated to about 22% with microsomal protein and the microsomal fraction to about 15% with mitochondrial protein.

Protein was determined as described by Lowry *et al.* (1951). The protein content of the mitochondrial fraction was 1.6mg/ml and of the microsomal fraction 1.2mg/ml. The standard used was crystallized bovine plasma albumin obtained from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.

Incubation procedures and analysis of extracts from incubation mixtures

Incubation mixtures consisted of 6.0ml of each fraction, 500μl of the homogenizing medium containing 10μmol of NADPH or an NADPH-generating system (Einarsson & Johansson, 1969), and 1.2μmol of sodium [24-^{14}C]cholate or [24-^{14}C]-deoxycholate.

Boiled whole homogenate was prepared by heating the whole homogenate for 5min in a boiling-water bath. After cooling it was used for the control incubation. Homogenizing medium was used as the control with subcellular fractions.

Incubations were performed at 28°C, except those with the NADPH-generating system, which were at 32°C, for 60min in air with mechanical shaking. The reactions were terminated by the addition of 20vol. of chloroform/methanol (2:1, v/v). The resulting precipitate was filtered off and 0.9% (w/v) NaCl solution (one-fifth of the total volume) was added to the filtrate, and the mixture shaken.

On separation the resulting chloroform phase was washed with 0.9% NaCl solution and the washings were combined. The combined aqueous phase was heated to remove organic solvents, diluted with 10ml of water, acidified with HCl to pH1 and twice extracted with 20ml portions of butan-1-ol. The combined butanol extract was washed three times with butan-1-ol-saturated water and evaporated to dryness below 50°C.

The overall extraction procedure gave an average recovery of 73% of the radioactivity added to the incubation mixture.

The butanol extracts were dissolved in methanol (200μl) and subjected to t.l.c. in system 1.

Thin layers of silica gel were divided, with radioautograms as guides, into zones numbered from the origins. The gel in each zone was scraped off, eluted twice with methanol and the combined methanol eluate was evaporated to dryness in a stream of N_2.

Percentage conversion of the substrate was calculated from radioactivity found in each zone on the plates. Rechromatography of eluates of each zone used the following developing solvent systems: chloroform/ethanol/acetic acid/water (12:8:4:1, by vol.) for the eluate from zone 1, ethyl acetate/acetic acid/methanol (8:1:1, by vol.) for zone 2, S6 (cyclohexane/ethyl acetate/acetic acid, 7:23:3, by vol.) for zone 3, S4 (benzene/dioxan/acetic acid, 55:40:2, by vol.) for zones 4, 5, 6 and 7 and S11 (2,2,4-trimethylpentane/ethyl acetate/acetic acid, 5:5:1, by vol.) (Eneroth, 1963) for zones 8 and 9.

The methanol eluates from different zones, after rechromatography of eluates from the original zones, were evaporated to dryness in a stream of N_2. The residues were dissolved in methanol (25μl), ethyl acetate (5ml) was added and the mixture was washed with water and evaporated to dryness in a stream of N_2. Methylation of the methanol eluate thus obtained was carried out with diazomethane after addition of a carrier (up to 0.25mg). The methanol eluates and their methyl esters thus purified were used for identification by the isotope-dilution method (Thomas *et al.*, 1964).

Bile acids were revealed with I_2 vapour and in some experiments by spraying with 0.01% pyrene in light petroleum (b.p. 60–80°C) (Eastwood & Hamilton, 1967). The gel, marked under u.v. light in the latter case, was scraped off and used for radioactivity assay.

Radioactivity was determined in a Beckman automatic scintillation spectrometer (model 1650). The scintillation fluid used was a solution in ethanol (300ml) and toluene (700ml) of 2,5-diphenyloxazole (4g) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (100mg) (Ikawa *et al.*, 1972).

Results

Analysis of bile salts of snakes

The component bile acids of four snakes from the subfamily Viperinae are summarized in Table 1.

The identification of bile acids was based on the original t.l.c. and i.r. examination or on g.l.c. after hydrolysis.

All snake gall-bladder bile tested contained bitocholic acid, cholic acid, allocholic acid and 3α,7α,12α,-23ξ-tetrahydroxycholanic acid as the major components and 3α,7β,12α-trihydroxy-5β-cholan-24-oic acid and deoxycholic acid as minor components.

Two unidentified compounds A and B (R_F 0.14 and 0.25 in system 1) were observed. Neither compound showed change of R_F after treatment with diazomethane.

Table 1. *Bile acid composition of four boid snakes*

The bile salts (taurine conjugates) of the four snakes were hydrolysed and the resulting acids methylated. The methyl esters of the bile acids detected are labelled A–I in the Table corresponding to the following: A, unidentified compound A; B, unidentified compound B; C, methyl 3α,7α,12α,23ξ-tetrahydroxycholanate; D, methyl allocholate; E, methyl cholate; F, methyl 3α,7β,12α-trihydroxy-5β-cholan-24-oate; G, methyl bitocholate; H, methyl chenodeoxycholate; I, methyl deoxycholate. The evidence for identification was obtained by methods a–c which are as follows: a, analytical t.l.c.; b, g.l.c. of methyl esters of methyl trimethysilyl ethers of bile acids; c, i.r. spectroscopy of bile acids after crystallization or purification on silica-gel sintered plates. R_F values were calculated from t.l.c. on Kieselgel G, developed with benzene/propan-2-ol/acetic acid (15:5:1, by vol.). A 50μg sample of each specimen was applied to the starting point.

Species ...		*Bitis arietans*			*Bitis nasicornis*			*Vipera russelli*			*Vipera berus*		
Bile acid component	R_F of methyl ester	(μg)	(%)	Method	(μg)	(%)	Method	(μg)	(%)	Method	(μg)	(%)	Method
A	0.14	0.9*	2.0		0.2*	0.5		0.4*	1.4		0.7*	2.2	
B	0.25	1.0*	2.2		1.2*	3.0		0.5*	1.7		1.3*	4.0	
C	0.38	9.3	20.6	a, b, c	10.7	27.1	a, b, c	5.2	17.9	a, b, c	8.9	27.5	a, b, c
D	0.45	5.4	11.9	a, b, c	2.8	7.1	a, b	4.9	16.9	a, b, c	3.7	11.5	a, b, c
E	0.50	8.5	18.8	a, b, c	5.5	13.9	a, b, c	2.9	10.0	a, b, c	6.4	19.8	a, b, c
F	0.58	1.5	3.3	a, b, c	1.9	4.8	a, b	1.0	3.4	a, b	1.9	5.9	a, b
G	0.65	13.1	30.0	a, b, c	12.1	30.6	a, b, c	7.3	25.1	a, b, c	4.5	13.9	a, b, c
H	0.70	4.2	9.3	a, b	3.7	9.4	a, b	5.0	17.2	a, b	2.5	7.7	a, b
I	0.74	1.3	2.9	a, b	1.4	3.5	a, b	1.8	6.2	a, b	2.5	7.7	a, b

* Amounts calculated assuming that the compounds, detected on t.l.c., had the same densitometric characteristics as methyl 3α,7α,12α,23ξ-tetrahydroxycholanate.

Table 2. *Percentage conversion of sodium [24-^{14}C]cholate and [24-^{14}C]deoxycholate in whole homogenates of adder liver supplemented with NADPH*

R_F values were calculated from t.l.c. on Kieselgel G developed with benzene/propan-2-ol/acetic acid (15:5:1, by vol.).

	No. of zone	R_F values	Conversion (%)	Compounds found
Cholate	10	1.00–0.88	Trace	
	9	0.88–0.74	3.0	
	8	0.74–0.60	0.7	
	7	0.60–0.50	9.3	7α,12α-Dihydroxy-3-oxo-5β-cholan-24-oic acid
	6	0.50–0.41	2.5	7α,12α-Dihydroxy-3-oxo-4-cholen-24-oic acid
	5	0.41–0.33	66.8	Cholic acid
	4	0.33–0.27	10.8	3β,7α,12α-Trihydroxy-5β-cholan-24-oic acid
	3	0.27–0.14	4.5	
	2	0.14–0.03	3.1	3α,7α,12α,23ξ-Tetrahydroxy-5β-cholan-24-oic acid
	1	0.03–0.00	0.3	Taurine conjugates
Deoxycholate	10	1.00–0.94	0.8	
	9	0.94–0.84	0.5	
	8	0.84–0.75	3.8	12α-Hydroxy-3-oxo-5β-cholan-24-oic acid
	7	0.75–0.64	82.1	Deoxycholic acid
	6	0.64–0.54	Trace	
	5	0.54–0.44	9.3	3α,7β,12α-Trihydroxy-5β-cholan-24-oic acid
	4	0.44–0.28	1.3	
	3	0.28–0.14	0.6	Bitocholic acid
	2	0.14–0.03	0.5	
	1	0.03–0.00	0.3	Taurine conjugates

Incubations of sodium [24-^{14}C]cholate with adder whole liver homogenates

Radioautographs of butan-1-ol extracts from incubation mixtures showed several radioactive zones, and the percentage conversion of substrate into each metabolite is summarized in Table 2. Rechromatography of the eluate from zone 1 showed 18.5% of the total radioactivity found in the zone in the band of taurocholate added as carrier.

Radioautograms of the eluates of zone 2 showed three bands, and the ratio of radioactivity of these bands was 4:1:1 in descending order of R_F values. The band with the largest R_F value corresponded to 3α,7α,12α,23ξ-tetrahydroxycholanic acid; however, the other two bands remained unidentified. The eluate from the band corresponding to 3α,7α,12α,23ξ-tetrahydroxycholanic acid was methylated and subjected to isotope dilution; crystallization to constant specific radioactivity confirmed the presence of 3α,7α,12α,23ξ-tetrahydroxycholanic acid.

The eluate from zone 3 was methylated and rechromatographed. The band on the resulting radioautogram did not coincide with that of methyl bitocholate (R_F 0.62 in solvent system 1).

A radioactive band of the eluate of zone 4 had the same R_F value in system 1 as allocholic acid and 3β,7α,12α-trihydroxy-5β-cholan-24-oic acid, but different from that of phocaecholic acid. In system S4, however, the radioactive band corresponded well to 3β,7α,12α-trihydroxy-5β-cholan-24-oic acid (R_F 0.48), but not to that of allocholic acid (R_F 0.39).

The band in the radioautogram of the eluate from zone 6 did not coincide with that of 3α,12α-dihydroxy-7-oxo-5β-cholan-24-oic acid or 3α,7α-dihydroxy-12-oxo-5β-cholan-24-oic acid but agreed well with 7α,12α-dihydroxy-3-oxo-4-cholen-24-oic acid. The eluates from zones 5 and 7 were identified as cholic acid and 7α,12α-dihydroxy-3-oxo-5β-cholan-24-oic acid respectively. The identity of these three compounds was confirmed, after addition of the appropriate carrier, by crystallization to constant specific radioactivity.

Incubation of sodium [24-^{14}C]deoxycholate with whole liver homogenates

The results of these incubations are summarized in Table 2.

The eluate from zone 1 was subjected to rechromatography by t.l.c. and the resulting radioautogram showed that radioactivity in the band of taurodeoxycholate was 14.9% of the total radioactivity of zone 1. Methylated eluate from zone 2 did not correspond to methyl 3α,7α,12α,23ξ-tetrahydroxycholanate. The radioautogram of methylated eluate from zone 3 showed two bands, one of which corresponded to methyl bitocholate and the other was a compound with R_F 0.25 in system S4. The ratio of radioactivity found in the bands corresponding to methyl bitocholate and the unidentified compound was about 3:1. The presence of methyl bitocholate was confirmed by isotope dilution and crystallization to constant specific radioactivity. The radioautogram of the eluate from zone 4 showed a radioactive band that was not identified with 3β,7α,12α-trihydroxy-5β-cholan-24-oic acid. That from zone 5 showed a band coincident with 3α,7β,12α-trihydroxy-5β-cholan-24-oic acid in both solvent systems 1 and S4. Eluates from zones 7 and 8 were identified as deoxycholic acid and 12α-hydroxy-3-oxo-5β-cholan-24-oic acid respectively after addition of carrier and crystallization to constant specific radioactivity.

Table 3. *Percentage conversion of sodium [24-^{14}C]cholate and [24-^{14}C]deoxycholate in subcellular fractions of adder liver supplemented with NADPH*

	No. of zone	Conversion (%)			Compounds found
		Microsomal	Mitochondrial	Supernatant	
Cholate	10	1.1	Trace	1.1	
	9	Trace	Trace	Trace	
	8	5.7	1.1	Trace	
	7	0.4	2.3	3.4	7α,12α-Dihydroxy-3-oxo-5β-cholan-24-oic acid
	6	1.2	0.1	0.1	7α,12α-Dihydroxy-3-oxo-4-cholen-24-oic acid
	5	78.0	91.5	95.3	Cholic acid
	4	1.2	0.1	Trace	
	3	6.3	2.8	0.1	
	2	6.2	2.3	Trace	3α,7α,12α,23ξ-Tetrahydroxy-5β-cholan-24-oic acid
	1	0.6	Trace	Trace	
Deoxycholate	10	0.3	0.4	0.3	
	9	1.9	3.0	1.0	
	8	9.1	8.7	4.5	12α-Hydroxy-3-oxo-5β-cholan-24-oic acid
	7	72.5	78.8	92.7	Deoxycholic acid
	6	0.4	Trace	Trace	
	5	10.1	5.2	Trace	3α,7β,12α-Trihydroxy-5β-cholan-24-oic acid
	4	0.4	0.5	0.2	
	3	2.8	1.3	0.3	Bitocholic acid
	2	1.5	0.1	Trace	
	1	1.8	1.1	1.8	

Table 4. *Percentage conversion of sodium [24-^{14}C]cholate and [24-^{14}C]deoxycholate in subcellular fractions of adder liver supplemented with an NADPH-generating system*

	No. of zone	Conversion (%)				Compounds found
				Supernatant		
		Microsomal	Mitochondrial	+Microsomal	+Mitochondrial	
Cholate	10	2.9	0.1	3.4	1.5	
	9	3.9	1.1	3.8	2.7	
	8	2.2	0.9	2.9	2.3	
	7	46.8	3.1	38.0	47.8	7α,12α-Dihydroxy-3-oxo-5β-cholan-24-oic acid
	6	5.8	2.4	14.2	7.5	7α,12α-Dihydroxy-3-oxo-4-cholen-24-oic acid
	5	35.7	92.6	28.8	32.6	Cholic acid
	4	0.6	Trace	1.6	0.6	
	3	1.4	Trace	1.5	0.7	
	2	1.5	0.1	3.8	2.7	3α,7α,12α,23ξ-Tetrahydroxy-5β-cholan-24-oic acid
	1	Trace	0.1	2.0	0.6	
Deoxycholate	10	4.4	Trace			
	9	4.7	1.1			
	8	53.8	13.7			12α-Hydroxy-3-oxo-5β-cholan-24-oic acid
	7	23.0	76.3			Deoxycholic acid
	6	1.5	1.7			
	5	8.4	3.3			3α,7β,12α-Trihydroxy-5β-cholan-24-oic acid
	4	1.0	1.0			
	3	1.2	1.1			Bitocholic acid
	2	1.2	0.7			
	1	0.6	0.6			

Incubations of sodium [24-^{14}C]cholate and [24-^{14}C]-deoxycholate with subcellular fractions of adder liver supplemented with NADPH

Percentage conversion of the substrate was calculated in the same way as for the whole homogenate and is summarized in Table 3.

Incubations of sodium [24-^{14}C]cholate and [24-^{14}C]-deoxycholate with subcellular fractions of adder livers supplemented with an NADPH-generating system

The results are listed in Table 4. In the incubation of sodium [24-^{14}C]cholate with the microsomal fraction, about 47% was converted into 7α,12α-dihydroxy-3-oxo-5β-cholan-24-oic acid, whereas in the mitochondrial fraction most of the deoxycholate remained unchanged. The formation of a small amount of 3α,7α,12α,23ξ-tetrahydroxycholanic acid was observed in the microsomal fraction.

Addition of the supernatant fraction to the microsomal and mitochondrial fractions considerably enhanced the formation of 7α,12α-dihydroxy-3-oxo-5β-cholan-24-oic acid and polar compounds.

Sodium [24-^{14}C]deoxycholate incubated with the microsomal fraction was mainly converted into 12α-hydroxy-3-oxo-5β-cholan-24-oic acid and 3α,7β,12α-trihydroxy-5β-cholan-24-oic acid, and the formation of bitocholic acid was observed to a small extent.

Discussion

Methodology

Purification of methyl esters of bile acids, which were eluted from layers of Kieselgel G or H (type 60), on silica-gel sintered plates was of great advantage in identification of bile acids by i.r. spectroscopy because it eliminated absorption due to silica. In some cases non-crystalline eluates also gave satisfactory i.r. spectra. The recoveries of the samples were constantly 94–96% and g.l.c. showed no artifacts. The quantitative assessment of bile acids of snakes was based on the original t.l.c., since it was fairly difficult to determine quantitatively bile acids ranging from monohydroxylated to tetrahydroxylated by g.l.c.

Biological

Both qualitatively and quantitatively bile acids from the four snakes, *B. arietans*, *B. nasicornis*, *V. russelli*

and *V. berus*, were similar to each other, and C-23-hydroxylated bile acids comprised 41–57% of the total (Table 1). The existence of bitcholic acid was not confined to the genus *Bitis* and cholic acid, and allocholic acid were found in all four snakes.

3α,7β,12α-Trihydroxy-5β-cholan-24-oic acid in gall-bladder bile is thought to be a secondary bile acid and may be formed by snake liver enzymes capable of 7β-hydroxylation of deoxycholic acid, or it may be synthesized by intestinal micro-organisms, as is almost certainly the case in human faeces (Eneroth *et al.*, 1966). The existence of deoxycholic acid suggests strongly that an enterohepatic circulation takes place in snakes.

The metabolites found on incubation of sodium cholate and deoxycholate with adder whole liver homogenates supplemented with NADPH indicate that adder liver contains systems for C-23- and C-7β-hydroxylation and 3α- and 3β-hydroxy steroid oxidoreductases. Under the experimental conditions deoxycholic acid was not hydroxylated at C-7α, in contrast with rats and mice, which have liver enzymes that catalyse such 7α-hydroxylation of deoxycholic acid (Bergström & Gloor, 1954, 1955). On the other hand 7β-hydroxylation of deoxycholic acid was observed. A similar 7β-hydroxylation of 3β-hydroxy-5-cholen-24-oic acid has been found in rabbit liver homogenates in the presence of NADPH and molecular oxygen (Yamasaki *et al.*, 1967).

In the case of C-23-hydroxylation of cholic acid and deoxycholic acid by the mitochondrial fraction, part of the observed hydroxylation results from contamination with the microsomal fraction. According to measurements of microsomal glucose 6-phosphatase activity the mitochondrial fraction was contaminated to about 22% with the microsomal fraction. If the microsomal C-23-hydroxylase activity sediments with glucose 6-phosphatase activity, about 59% of the C-23-hydroxylation of cholic acid and about 47% of that of deoxycholic acid would be due to the microsomal contamination.

The evidence suggests that C-23-hydroxylation of cholic acid and deoxycholic acid was also carried out by the action of a C-23-hydroxylase in the mitochondrial fraction itself. To judge from the percentage conversion of cholic acid into 3α,7α,12α,23ξ-tetrahydroxycholanic acid per mg of protein of the microsomal fraction (0.43%/mg) and of the mitochondrial fraction (0.20%/mg), the activity of mitochondrial C-23-hydroxylase may be about half that of the microsomal enzyme.

In the supernatant fraction cholic acid was mainly converted into 7α,12α-dihydroxy-3-oxo-5β-cholan-24-oic acid, and deoxycholic acid was mainly converted into 12α-hydroxy-3-oxo-5β-cholan-24-oic acid. These results suggest that 3α-hydroxy steroid oxidoreductase is located in the supernatant fraction of adder liver. This is the case in rat liver (Tomkins, 1956; Koide, 1969). The results suggest that a C-23-hydroxylase system in adder liver is mainly located in the microsomal fraction and partly in the mitochondrial fraction. The evidence is of interest in comparison with the C-26-hydroxylation of various C_{27} steroids by the microsomal and mitochondrial fractions of the rat, requiring NADPH as a cofactor (Björkhem & Gustafsson, 1973).

In incubations of sodium cholate or deoxycholate with the subcellular fractions supplemented with an NADPH-generating system, conversion ratios of the substrate to each metabolite were considerably different from those in the subcellular fractions supplemented with NADPH. The difference may result from inefficiency in generating NADPH, since the incubation was carried out at 32°C, away from the optimal temperature for isocitrate dehydrogenase.

The results of the present investigation imply the following possible pathways for the biosynthesis of the C-23-hydroxylated bile acids. (1) C-23-hydroxylation of cholic acid mainly by the microsomal fraction of snake liver, followed by C-7α-dehydroxylation of the resulting 3α,7α,12α,23ε-tetrahydroxycholanic acid by intestinal micro-organisms; (2) C-7α-dehydroxylation of cholic acid by intestinal micro-organisms, followed by C-23-hydroxylation of the resulting deoxycholic acid mainly by the microsomal fraction of snake liver.

It seems most likely that the major biosynthetic pathway of bitocholic acid is (1), since small amounts of deoxycholic acid were contained in the snake biles examined and the formation of bitocholic acid from deoxycholic acid appears to be about five times less than that of 3α,7α,12α,23ξ-tetrahydroxycholanic acid from cholic acid.

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