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Comparative Studies of 'Bile Salts'

5. BILE SALTS OF CROCODYLIDAE

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The Crocodylidae are reptiles which are believed to have persisted without much alteration for a very long period. Accordingly it was expected, on the hypotheses put forward in these studies, that their bile salts would show primitive characteristics. A brief report of work on *Alligator mississippiensis* has already appeared (Haslewood, 1952*a*) and details of this, together with some findings in two other species, namely the spectacled cayman (*Caiman crocodilus*) and the Nile crocodile (*Crocodylus niloticus*) are now presented.

RESULTS

The work described shows that the bile acids of each of the three species examined are present in similar complex mixtures. Crystalline material was easily obtained, but this, too, was very impure. By chromatography on alumina it proved in every case simple to isolate the ethyl ester of an acid $C_{27}H_{46}O_5$, named 'trihydroxycoprostanic acid'; this was further characterized as the parent acid and its methyl ester. The dehydro derivatives obtained after oxidation with chromic acid contained three carbonyl groups in the molecule. These groups could be removed by Kishner-Wolff reduction, which gave the 'stem acid', coprostanic acid, $C_{27}H_{46}O_2$; and this has been prepared by partial synthesis from cholanic acid (Bridgwater & Haslewood, 1952).

Through the kindness of Prof. T. Shimizu of the University of Okayama Medical School, Japan, it has been possible to show that trihydroxycoprostanic acid is identical with the 'α-trihydroxybisnorsterocholanic acid', isolated by Kurauti & Kazuno

(1939) from the bile of the frog *Rana catesbiana*. This finding suggests a change in nomenclature, which is discussed below.

EXPERIMENTAL

General. All melting points are uncorrected. Al_2O_3 was obtained from Hopkin and Williams and neutralized (Shoppee, 1949). Micro-analysis was done by Weiler and Strauss, Oxford. Optical rotations were determined in a 1 dm. microtube. 20% CrO_3 is a solution made by dissolving CrO_3 (20 g.) in the minimal amount of water and making up to 100 ml. with acetic acid. H-test = Hammersten's HCl test (Haslewood, 1943). Light petroleum, 'b.p. 40–60°'.

Fractionation of bile acids

The methods described by Haslewood & Wootton (1950) were followed. In all species, liquors from the hydrolysis of the bile salts were tested with $BaCl_2$ for SO_4^{2-}. Only small amounts of this ion were detected.

Alligator mississippiensis. Seven gall bladders, from animals 4–8 ft. long, yielded about 16·7 g. of light-brown hygroscopic bile salts. These proved somewhat resistant to hydrolysis, and after experiments the following method was adopted: a solution of the bile salts (1 g.) in water (5 ml.) with NaOH (5 ml. of 5N) was sealed in a small metal bomb and heated at 118–120° for 16 hr. Extraction of the contents of the cooled bomb with water gave a clear brown solution which was treated with excess of dilute HCl and of solid NaCl. After some hours, the precipitated gummy bile acids were collected and washed. Part of the precipitated bile acids tended to dissolve on washing, and to be re-precipitated with the NaCl-containing liquors. This material did not appear to be unchanged bile salts, and was similar to the ethyl acetate-insoluble precipitate (below). The main washed bile acid precipitate was dissolved in ethanol and the solution evaporated. The residue, with ethyl acetate, gave an insoluble gummy solid which was filtered off and kept. This material was not, apparently, altered on further heating with alkali as above. The ethyl acetate filtrate was evaporated to small bulk and set aside for crystallization.

The crystals were collected after 4–5 days at 0–5°, washed with cold ethyl acetate and dried. The yield from six such experiments was 0·14–0·19 g. (average 0·17 g.). This material (1·5 g.) was recrystallized once from ethyl acetate and gave short white needles (0·8 g.), m.p. 160–165°. Ethylation with diazoethane gave esters (0·59 g.) and unesterified acids which were extracted from ethereal solution with dilute ammonia. The esters (0·59 g.) left on evaporation of the washed ether were dissolved in benzene and separated on Al_2O_3 (6 g.) in a column. Benzene (1245 ml.) eluted a total of 0·35 g. which crystallized on standing with light petroleum. Ether (105 ml.) then eluted 0·15 g. and ethanol (40 ml.) 0·07 g. from the column. Total eluted, 0·57 g. The 0·35 g. of material eluted with benzene was *ethyl trihydroxycoprostanate*; the purest samples obtained had m.p. 143·5–144·5°, and crystallized from light petroleum/benzene in small colourless leaflets or flat needles. $[\alpha]_D^{21°} + 28 \pm 2°$ in ethanol (*c*, 1·7). (Found: C, 72·4; H, 10·2. $C_{29}H_{50}O_5$ requires C, 72·8; H, 10·5%.) This substance was more easily eluted from Al_2O_3 than ethyl cholate. Further samples of this ester were obtained by re-chromatography of the material eluted with ether (elution with benzene removed the ethyl trihydroxycoprostanate) and also, by the same procedure, after re-esterification of the acid not esterified by the first treatment with diazoethane.

In general, elution of the ethyl esters from Al_2O_3 with benzene removed most of the ethyl trihydroxycoprostanate, and with ether and then ethanol material was obtained which was a mixture. Such material was recrystallized from ethanol/water to give long white needles, m.p. 200–203°, which were still not sufficiently pure to give satisfactory analytical figures. (Found: C, 71·5; H, 10·6%.)

The ethyl acetate liquors from which the crystalline acids had been filtered were evaporated and the gummy residue (4·35 g.) was twice treated with diazoethane and separated into esters and unesterified acids as described above. The combined ester fraction (3·0 g.) was dried over H_2SO_4 *in vacuo*; it was then dissolved in ethanol (30 ml.) with Girard's reagent 'T' (1·0 g.) and acetic acid (3 ml.). The mixture was boiled under reflux for 80 min.; then partially neutralized and separated into ketones (0·062 g.) and non-ketones (2·85 g.) in the usual way. The ketonic fraction, on separation on Al_2O_3 (1 g.) in a column, gave a small amount of crystalline material. The non-ketones, treated similarly, gave no easily identifiable crystalline material.

Caiman crocodilus. One gall bladder gave about 2·5 g. hygroscopic bile salts which were treated exactly as those of the alligator. The crude ethyl acetate crystals from the bile salts (2 g.) weighed 0·36 g., and had m.p. 169–172°. These were dissolved in ethanol (7 ml.) with H_2SO_4 (0·1 ml.) and the solution left at approx. 22° for 16 hr. Dilution with $NaHCO_3$ solution precipitated the crude ethyl esters (0·34 g.), which were filtered off. (The filtrate on acidification gave a little unesterified acid, which crystallized on standing.) The esters (0·34 g.) were separated on Al_2O_3 as described above and, on elution with benzene, yielded ethyl trihydroxycoprostanate (0·19 g.), m.p. 138–140° (not depressed by mixture with the ester from alligator bile), together with (probably) more hydroxylated esters (0·14 g.) eluted with ether and ethanol. Ethyl triketocoprostanate (see below) from the above ester (m.p. 138–140°) had m.p. 203–206°; coprostanic acid (see below), m.p. 101–104°, was prepared from the triketo ester.

Crocodylus niloticus. One gall bladder gave about 0·7 g. of light-brown hygroscopic bile salts. These were treated as described above. The crude crystals (0·12 g.) from ethyl acetate had m.p. 162–168°. The ethyl trihydroxycoprostanate, prepared as above, had m.p. 138–140° (not depressed by mixture with the ester from alligator bile); the derived ethyl triketocoprostanate had m.p. 203–204° and the coprostanic acid had m.p. 100–103°.

Trihydroxycoprostanic acid and its derivatives

Substances which are probably identical with those previously described by Kurauti & Kazuno (1939) or Mabuti (1941*b*) under different names are indicated by an asterisk.

*Trihydroxycoprostanic acid**. Ethyl trihydroxycoprostanate (100 mg.), ethanol (1 ml.) and KOH solution (0·1 ml. of 40% w/v) were gently boiled together under reflux for 15 min. The solution was diluted with water and acidified with HCl. The precipitated acid was collected, washed with water and dissolved in ethanol. Evaporation of the filtered solution under N_2 in a test tube left a residue which was crystallized from ethyl acetate (1–2 ml.). *Trihydroxycoprostanic acid* formed short white needles which melted at 167–168° to a cloudy liquid which cleared

sharply at 174°. These apparently contained some solvent, or water, for it was necessary to dry to constant weight at 80° to obtain satisfactory analytical figures. (Found: C, 72·2; H, 10·5; equiv. by titration, 446. $C_{27}H_{46}O_5$ requires C, 72·0; H, 10·2%; mol.wt., 450.) Trihydroxycoprostanic acid crystallized from ether in hexagonal prisms (m.p. 171–173°) very similar to those obtainable from cholic acid. It gave a blue-green colour in the H-test.

Methyl trihydroxycoprostanate. A solution of the above acid (50 mg.) in methanol (1 ml.) with H_2SO_4 (1 drop) was allowed to stand at about 22° for 4 days. Dilution with aqueous $NaHCO_3$ gave the ester as a gum: esterification was apparently quantitative. The ester, in benzene, was eluted from Al_2O_3 (0·5 g.) with benzene and the product recrystallized from light petroleum/benzene, from which *methyl trihydroxycoprostanate* separated as small white needles, m.p. 153–155°. (Found: C, 72·5; H, 10·5. $C_{28}H_{48}O_5$ requires C, 72·4; H, 10·4%.)

Ethyl triketocoprostanate. Ethyl trihydroxycoprostanate (0·1 g.) in acetic acid (1·5 ml.) was treated with 20% CrO_3 (0·3 ml.), added gradually with efficient cooling in cold water (this was necessary to obtain a product of sharp melting point). After 10 min. at about 20°, the solution was diluted with water and the product isolated by filtration and thoroughly washed with water. It was crystallized from dilute ethanol, from which *ethyl triketocoprostanate* separated as fine colourless needles of m.p. 205–206°. (Found: C, 73·6; H, 9·3. $C_{29}H_{44}O_5$ requires C, 73·7; H, 9·3%.)

Methyl triketocoprostanate. This was similarly prepared from methyl trihydroxycoprostanate and crystallized from dilute ethanol in small colourless needles which had m.p. 222–224°. (Found: C, 72·9; H, 9·1. $C_{28}H_{42}O_5$ requires C, 73·3; H, 9·2%.)

Triketocoprostanic acid.* This was prepared by hydrolysis of ethyl triketocoprostanate and crystallized from benzene/acetone as small white needles, which had m.p. 227–228° (decomp.). (Found: C, 73·2; H, 9·1. $C_{27}H_{40}O_5$ requires C, 73·0; H, 9·0%.)

Coprostanic acid. Ethyl triketocoprostanate (34 mg.) was added to a solution, in a small metal bomb, of Na (50 mg.) in ethanol (2 ml.) with hydrazine hydrate (0·1 ml. of 90–95%). The bomb was sealed and heated at 200–207° for 4 hr. The contents of the cooled bomb were then washed out with water and acidified with HCl. The solid which separated was collected, washed with water and dissolved in methanol. The filtered solution was warmed and diluted to cloudiness with water. On standing, colourless needles (16 mg.) separated. These could be recrystallized from aqueous methanol or aqueous acetone from which *coprostanic acid* separated as long glistening colourless needles which had m.p. 103·5–105·5°; $[\alpha]_D^{21°} + 28 \pm 2°$ in $CHCl_3$ (c, 0·7). (Found: C, 81·1; H, 11·0. $C_{27}H_{46}O_2$ requires C, 80·5; H, 11·5%.) The Kishner-Wolff reduction had to be carried out with careful attention to details of quantities, especially of Na, in order to obtain a product from which crystals could be isolated.

Comparison of trihydroxycoprostanic acid and 'α-trihydroxybisnorsterocholanic acid'

Prof. T. Shimizu kindly sent the author, at his request, 0·1 g. of the acid 'm.p. 172°' from the bile of *Rana catesbiana*. Shimizu's acid had m.p. 166–169·5°. The mixed melting point with trihydroxycoprostanic acid (m.p. 167–168°) was 163–170°. Shimizu's acid (50 mg.) was dissolved in ethan (1 ml.) with H_2SO_4 (1 drop) and the solution kept at about 21° for 2 days. Dilution with aqueous $NaHCO_3$ precipitated the ester as a semi-solid (the $NaHCO_3$ liquors gave a cloudy solution on acidification). The ester was dried by evaporation with ethanol, dissolved in benzene and absorbed on to Al_2O_3 (0·5 g.) in a slow-moving column. Benzene (40 ml.) eluted 30·7 mg., ether (25 ml.) 7·8 mg. and ethanol (25 ml.) 10·4 mg. Total eluted, 48·9 mg. The 30·7 mg. from the benzene eluate crystallized, on treatment with light petroleum, as highly purified ethyl trihydroxycoprostanate, m.p. 143·5–144·5°, not depressed by the ester (m.p. 140–142°) from alligator's bile. The ether eluate yielded a rather less pure specimen of ethyl trihydroxycoprostanate and the ethanol eluate was partially crystalline. Hence, at least about 45 mg. (90%) of Shimizu's product must have been trihydroxycoprostanic acid. Ethyl triketocoprostanate from Shimizu's trihydroxy ester had m.p. 200–202°, not depressed by material (m.p. 205–206°) prepared from alligator's bile.

DISCUSSION

Biological

The presence of a C_{27} bile acid in Crocodylidae is in accordance with the view that the family is primitive in an evolutionary sense. It is of particular interest that this acid is identical with one from the frog *Rana catesbiana*, the bile of which species contains as its chief constituent a C_{27} or C_{28} alcohol, conjugated with sulphate (Kurauti & Kazuno, 1939), as in the case of *R. temporaria* (Haslewood, 1952*b*). These findings suggest that trihydroxycoprostanic acid is not to be looked upon as a bile acid characteristic of Crocodylidae or, indeed, of any other family, but rather as an early type of bile acid which may at one time have been widely distributed in the animal kingdom, as is cholic acid amongst 'modern' vertebrates. If this is correct we may expect to find trihydroxycoprostanic acid and its relatives on many occasions in the bile of what are now regarded as 'primitive' creatures. Its presence in minor amounts in frog's bile may mean that the original alcohol-sulphate bile salt type in these animals is giving way to the now common acid-taurine type of conjugate.

Bile acids with more than twenty-four carbon atoms in the molecule have been encountered on several occasions: a list is given in Table 1. A study of this supports the view that such acids are characteristic of the bile of the more primitive vertebrates.

Chemical

Trihydroxycoprostanic acid shows an obvious general similarity to cholic acid, and this similarity even extends to one of the crystalline forms. The Hammarsten reaction is much less obvious than with cholic acid, and there is also marked resistance to ethylation. Mabuti (1941*b*) has degraded 'α-trihydroxybisnorsterocholanic acid' to cholic acid. This proves the position and configuration of the

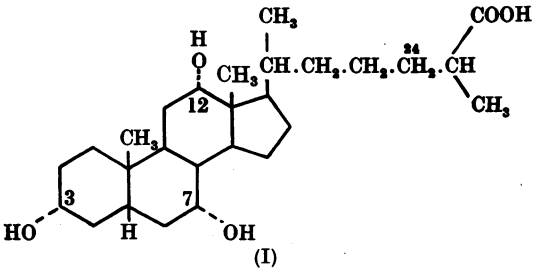
Table 1. *Bile acids with molecules containing more than twenty-four carbon atoms*

Acid	Melting point (°)	Melting point of dehydro acid (°)	Species	Reference	Remarks
$C_{27-28}H_{46-48}O_6$	255	175	A shark	Ohta (1939*a*)	—
Tetrahydroxynorsterocholanic, $C_{27}H_{46}O_6$	217	—	Teleosts:		OH at $C_{(3)}C_{(6)}C_{(12)}$ and $C_{(24)}$. Gives a positive Hammarsten reaction. Converted to *allo*cholanic acid, 12-ketocholanic acid and hyodeoxycholic acid (Ohta, 1939*b*; Isaka, 1940)
			Pelteobagrus nudiceps	Ohta (1939*b*)	
			Tetrodon porphyleus	—	
			'Bari' fish	Isaka & Azato (1940)	
			Inimicus japonicus	Mabuti (1941*a*)	
Trihydroxybufosterocholenic,* $C_{28}H_{46}O_5$	160	269	Japanese toad, *Bufo vulgaris*	Shimizu & Oda (1934)	Degraded to bisnorcholic acid (Shimizu & Kazuno, 1936)
Trihydroxy*iso*sterocholenic,* $C_{26}H_{44}O_5$	227	214	Japanese toad, *B. vulgaris*	Shimizu & Kazuno (1936)	Degraded to bisnorcholic acid (Shimizu & Kazuno, 1937)
α-Trihydroxybisnorsterocholanic,*† $C_{26}H_{44}O_5$	172	231	Frog, *Rana catesbiana*	Kurauti & Kazuno (1939), Mabuti (1941*b*)	Degraded to cholic acid (Mabuti, 1941*b*)
β-Trihydroxybisnorsterocholanic,* $C_{26}H_{44}O_5$	190–195	Identical with the above	Frog, *R. catesbiana*	Mabuti (1941*c*)	
Tetrahydroxysterocholanic, $C_{27-28}H_{46-48}O_6$	150	215	Turtle, *Amyda japonica*	Yamasaki & Yuuki (1936)	Easily forms a lactone, m.p. 208°. Degraded to bisnorcholic acid and other 3:7:12-trihydroxy steroids (Kanemitu, 1942)
			Tortoise, *Emys orbicularis*	Kim (1939)	
Tetrahydroxy*iso*sterocholanic, $C_{27-28}H_{46-48}O_6$	205	188	*Amyda japonica*	Suganami & Yamasaki (1942)	Easily forms a lactone, m.p. 220°
Trihydroxycoprostanic,† $C_{27}H_{46}O_5$	173	228	*Alligator mississippiensis*, *Caiman crocodilus*, *Crocodylus niloticus*	Present work	Carbon skeleton of coprostane; carboxyl at $C_{(26)}$ (Bridgwater & Haslewood, 1952)
Varanic, $C_{27}H_{46}O_6$	120 (hydrate)	—	Lizard, *Varanus niloticus*	Haslewood & Wootton (1950)	—

* Not the major constituents of the bile salts.

† Identical (present work).

nuclear hydroxyl groups, and, together with the work of Bridgwater & Haslewood (1952), leaves little doubt that trihydroxycoprostanic acid is 3α:7α:12α-trihydroxycoprostanic acid (I).



(I)

Note on nomenclature. Kurauti & Kazuno's name 'trihydroxybisnorsterocholanic' for the acids from *R. catesbiana* is based on a 'sterocholanic acid', $C_{26}H_{48}O_2$, of partially known structure. It is now proposed to apply a nomenclature based on coprostanic acid (a substance of known structure) to C_{27} bile acids which can be converted to this compound. Prof. T. Shimizu and Prof. T. Kazuno have generously agreed to this change in the case of 'α-trihydroxybisnorsterocholanic acid' which is therefore to be called 'trihydroxycoprostanic acid'.

SUMMARY

1. A mixture of crystalline acids was readily isolated after alkaline hydrolysis of the bile salts of three species of Crocodylidae. After ethylation of this mixture the esters could be separated on alumina. This process gave the ethyl ester of an acid, $C_{27}H_{46}O_5$, and a mixture of esters of (probably) more hydroxylated acids. The acid $C_{27}H_{46}O_5$ was prepared from the ethyl ester and has been called 'trihydroxycoprostanic acid'. The corresponding triketocoprostanic acid, $C_{27}H_{40}O_5$, and some of its derivatives were prepared. Ethyl triketocoprostanate on Kishner-Wolff reduction and hydrolysis gave the 'stem acid', coprostanic acid, $C_{27}H_{46}O_2$.

2. Trihydroxycoprostanic acid is identical with the 'α-trihydroxybisnorsterocholanic acid' isolated from the bile of *Rana catesbiana* by Kurauti & Kazuno (1939). Since this substance was degraded to cholic acid by Mabuti (1941*b*), trihydroxycoprostanic acid is almost certainly 3α:7α:12α-trihydroxycoprostanic acid. With the agreement of the Japanese workers, it is suggested that 'α-trihydroxybisnorsterocholanic acid' be referred to as 'trihydroxycoprostanic acid'.

The author thanks Prof. T. Shimizu for the 'α-trihydroxybisnorsterocholanic acid'; Dr W. H. Pearlman (Jefferson Medical College, Philadelphia) and the Sabine National Wildlife Refuge (Louisiana) for alligator bile; Dr L. C. Beadle (Makerere College, Uganda) for crocodile bile and Dr R. E. Rewell, Mr W. E. Lawrence and the Zoological Society of London for the bile of the cayman.

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