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Comparative Studies of Bile Salts

BILE SALTS OF STURGEONS (ACIPENSERIDAE) AND OF THE PADDLEFISH *POLYODON SPATHULA*: A NEW PARTIAL SYNTHESIS OF 5β-CYPRINOL

By G. A. D. HASLEWOOD and A. R. TAMMAR
Guy's Hospital Medical School, London, S.E. 1

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1. Bile salts of the sturgeons *Acipenser guldenstaedti* Brandt, *Acipenser stellatus* Pall and *Huso huso* L. and of the paddlefish *Polyodon spathula* Walbaum are shown to be closely similar, consisting mainly of taurocholate with minor amounts of tauroallocholate and the monosulphates of bile alcohols. The bile alcohols, comprising less than 10% of the bile salts, are mixtures with high proportions of substances resembling C_{27} tetrols and of C_{27} pentols, including 5β-cyprinol and (probably) 5α-cyprinol. 2. 5β-Cyprinol (3α,7α,12α,26,27-pentahydroxy-5β-cholestane) was made from cholic acid via 3α,7α,12α-triacetoxy-5β-cholan-24-ol in an overall yield of about 0·8%. 3. The chemical nature of chondrostean bile salts agrees with the systematic position of the fishes and suggests further correspondence between evolution at the morphological and molecular levels.

The sturgeons and paddlefish belong to an Order (Chondrostei) of bony fishes (Osteichthyes) whose affinities with other Orders are not well understood. Romer (1966), regarding them as 'degenerate survivors of the older actinopterygians', points out that their largely cartilaginous skeleton is a result of evolutionary loss of ossification and that certain bodily features, for example the fins and tail, are undoubtedly primitive.

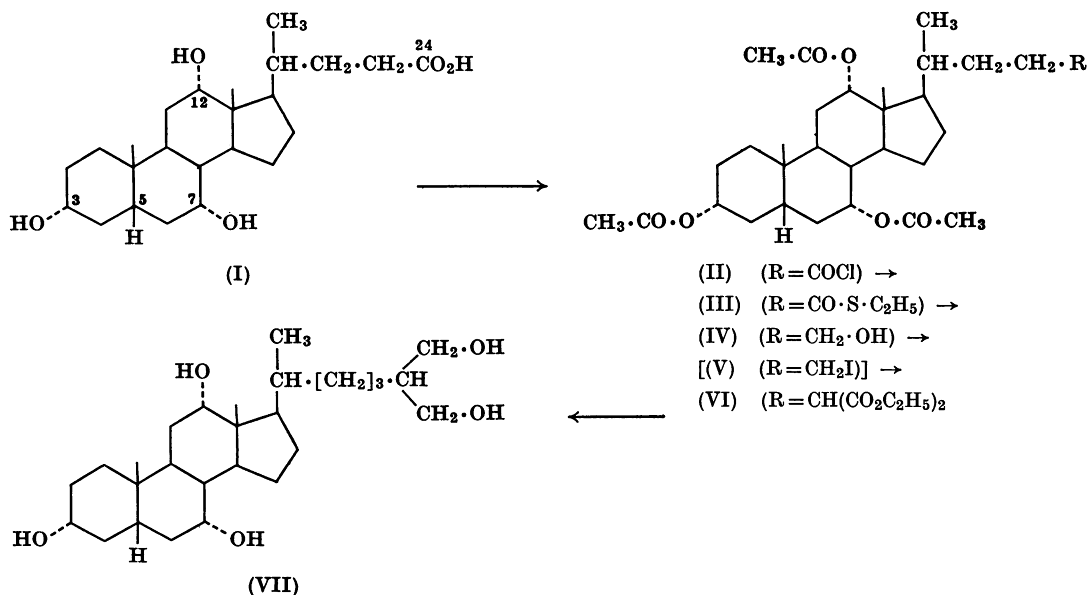
During our studies on bile salt chemistry we have generally observed a good correlation between the views of systematists, based chiefly on paleontology and morphology, and the chemical nature of the bile salts. We therefore thought that an examination of sturgeon and paddlefish bile might be of interest: the results of this, together with an improved partial synthesis of a chondrostean bile alcohol (5β-cyprinol), are now presented.

RESULTS

The bile salts of three sturgeon species, *Acipenser guldenstaedti* Brandt, *Acipenser stellatus* Pall and *Huso huso* L., all from Russia, closely resembled those of the North American paddlefish *Polyodon spathula* Walbaum. In all cases the principal constituent was taurocholate [the taurine conjugate of 3α,7α,12α-trihydroxy-5β-cholanoic acid (cholic acid, I)] together with an easily detectable proportion of tauroallocholate, its 5α-analogue. There was also a minor amount (probably less than 10% by wt.) of bile alcohol sulphates. The derived bile alcohols comprised an obviously complex mixture, not completely resolved, whose infrared spectrum showed that it contained substances whose molecules had the allocholic acid ring nucleus and others with the substituted ring structure of cholic acid. Very roughly half the alcohol mixture had the polarity on chromatograms of a C_{27} pentol, and from *H. huso* it proved possible to obtain, in a fair state of purity, a compound identified by its nuclear-magnetic-resonance (n.m.r.) spectrum as 5β-cyprinol (3α,7α,12α,26,27-pentahydroxy-5β-cholestane, VII) (Cross, Landis & Murphy, 1965). The properties of this sturgeon alcohol, temporarily called 'Acipenserol-A' (Cross *et al.* 1965), appeared not to correspond exactly to those given for 5β-cyprinol (Hoshita, Kouchi & Kazuno, 1963), and a sample of 5β-cyprinol kindly sent to us by Professor Kazuno was insufficient for direct comparison. We could not obtain 5β-cyprinol by the method of Hoshita *et al.* (1963) and therefore devised the following partial synthesis.

Cholic acid (I) was converted into triacetylcholyl chloride (II), which with ethanethiol in pyridine gave ethyl 3α,7α,12α-triacetoxy-5β-cholane-24-thiolate (III). Compound (III) with Raney nickel gave the triacetoxy-24-ol (IV), which, via the iodide (V) (not isolated), gave the malonic ester (VI). Reduction of compound (VI) with lithium aluminium hydride gave 5β-cyprinol (VII), m.p. 175–177° and $[\alpha]_D$ +35·5°, in an overall yield of about 0·8% (based on cholic acid).

5β-Cyprinol did not depress the melting point of



'Acipenserol-A' and Dr A. D. Cross reported that its n.m.r. spectrum was compatible with its identity as structure (VII) and as 'Acipenserol-A'. We conclude that the latter substance is indeed a not quite pure sample of 5β-cyprinol.

The availability of authentic 5β-cyprinol enabled us to compare its infrared spectrum with that of chimaerol [3α,7α,12α,24ζ,26(or 27)-pentahydroxy-5β-cholestane]. The spectra in potassium bromide differ only in the relative proportions of some of the bands, and, as remarked by Bridgwater, Briggs & Haslewood (1962), such spectra do not alone provide a reliable means of distinguishing between some pentols with the cholic acid nucleus. The infrared spectrum and properties of the substance of m.p. 171–173° made from cholic acid by Bridgwater *et al.* (1962) and thought by them to be probably an isomer of chimaerol ('26-deoxyscymnol') show that, in fact, it is 5β-cyprinol.

EXPERIMENTAL

General

Method and elementary analyses were as described by Bridgwater *et al.* (1962) and by Haslewood (1967).

Sturgeons' bile salts

Bile (100 ml.) of *A. guldenstaedti* Brandt, *A. stellatus* Pall and *Huso huso* L. was collected in ethanol (200 ml.) through the kindness of Professor V. A. Englehardt and brought from Moscow by Mrs Antoinette Pirie. The bile was further diluted with ethanol and the mixture filtered, evaporated and dried *in vacuo* over $CaCl_2$, giving bile salts (6·16, 12·35 and 17·10 g. respectively) as dark-brown, slightly hygroscopic, solids.

Alkaline hydrolysis. Bile salts (1 g.) of each species were heated at 115° for 17–19 hr. in a sealed metal bomb in 2·5 N-NaOH (10 ml.). The products each contained a solid, which was collected, washed with water and dissolved in ethanol. Evaporation left 'alkaline-hydrolysis neutral materials' (yields: from *A. guldenstaedti*, 11 mg.; from *A. stellatus*, 10 mg.; from *H. huso*, 18 mg.) as solids that gave weakly positive responses to the Hammarsten (HCl) test. On paper chromatography these fractions gave several spots; they were not further investigated. The alkaline liquors from each neutral fraction were treated with 2 N-HCl and NaCl (excess) and refrigerated. The bile acids were collected, washed with water and dissolved in ethanol. Evaporation left sturgeon bile acids as brown semi-solids (yields: from *A. guldenstaedti*, 0·57 g.; from *A. stellatus*, 0·57 g.; from *H. huso*, 0·64 g.). The acid aqueous liquors were treated with 0·5 M-$BaCl_2$ (10–20 ml.) giving $BaSO_4$ (from *A. guldenstaedti*, 71 mg.; from *A. stellatus*, 42 mg.; from *H. huso*, 40 mg.). The bile acids were boiled with ethyl acetate and the solutions filtered. Evaporation left residues that crystallized readily from ethyl acetate, giving hydrated crystals and an ethyl acetate-soluble fraction, obtained by evaporation of the liquors. Yields and melting points are given in Table 1. The crystalline bile acids were converted into ethyl esters in the usual way (see under 'General' above); yields were almost quantitative and the esters crystalline. Ethyl esters were recrystallized: as an example, *A. stellatus* ethyl esters (0·41 g.), m.p. 158–164°, after recrystallization from benzene (twice), aqueous ethanol, aqueous acetone and acetone–light petroleum (b.p. 40–60°), gave white needles (11 mg.), m.p. 192–198°, of a mixture of ethyl cholate and ethyl allocholate as described by Anderson & Haslewood (1962). No more than traces of bile acids other than cholic acid and allocholic acid were found by chromatography in any crystalline fraction; however, paper chromatography

Table 1. *Yields and melting points of bile acids from 1g. of sturgeons' bile salts, hydrolysed as described in the text*

Species	Bile acids from ethyl acetate (g.)	Ethyl acetate-soluble fractions (g.)
A. guldenstaedti	0·34 (m.p. 124–135°)	0·19
A. stellatus	0·47 (m.p. 183–186°)	0·10
H. huso	0·52 (m.p. 110°, then 180–185°)	0·09

in the Bush (1952) system A of the ethyl esters of the ethyl acetate-soluble fraction (Table 1) gave, in each case, a faint spot with the mobility of ethyl chenodeoxycholate (ethyl 3α,7α-dihydroxy-5β-cholanoate). The proportion of allocholic acid from *A. stellatus* may be very roughly estimated as at least 1·8% (by wt.) of the crystalline bile acids [7·5mg., i.e. 2:1 (w/w) of the 11mg. of ester described above, in 410mg. of total ethyl esters].

Dioxan–trichloroacetic acid cleavage. H. huso bile salts (4g.) were acetylated and cleaved by 40% (w/w) dioxan–trichloroacetic acid as described by Haslewood (1964). The ether-soluble neutral product was hydrolysed by boiling under reflux for 1hr. with methanolic 0·5N-NaOH. Removal of solvent with N_2 left a solid residue, which was mixed with water, collected on a filter and washed with water. The collected material was filtered in methanol; evaporation left '*H. huso* bile alcohols' as a brown 'glass' [0·16g.; approx. 4% (by wt.) of bile salts], which on paper chromatograms in the system di-isopropyl ether–light petroleum (b.p. 80–100°)–acetic acid–water (6:4:7:3, by vol.) gave spots corresponding to 5α-cyprinol as well as to more and less polar substances.

H. huso bile alcohols (84mg.) were partially purified by filtration after warming with acetone, in which a little apparently polymeric material remained insoluble. Evaporation left a residue (80mg.) that, from acetone, gave impure crystals (approx. 20mg.), m.p. 180–209°, giving a purple Hammarsten test. This material was recombined with the acetone-soluble fraction and part (66mg.) of the total product was separated on Celite (10g.) as described by Bridgwater, Haslewood & Watt (1963) in the system benzene–light petroleum (b.p. 80–100°)–ethanol–water (5:2:5:2, by vol.). Stationary phase (total 0·7ml.) was used to put the product on to the column, and 84 portions (each of 2ml.) of effluent moving phase were collected. Three main fractions were obtained: a brown gum (fraction A, 19mg., eluted by 0–28ml. of effluent), a partially crystalline solid mixture (fraction B, 25mg., eluted by 30–62ml. of effluent) and an impure solid (fraction C, 29mg., eluted by 84–142ml. of effluent). Fraction A apparently did not consist of bile alcohols. Fraction B included crystals (4mg., eluted by 32–36ml. of effluent), m.p. about 190–197°, whose infrared spectrum in KBr showed a pattern of bands between 9·0 and 11·2mμ, such as to suggest that it consisted of a mixture of some substances with the cholic acid nucleus and others with that of allocholic acid (Haslewood, 1967). On paper and thin-layer chromatograms fraction B gave several spots, all with about the polarity in the solvent systems used that would be expected of C_{27} steroid tetrols.

The highest concentrations of fraction C were eluted by 100–120ml. of effluent and material (13mg.) from this portion of moving phase gave, from ethyl acetate, crystals that lost solvent at about 175°, recrystallized and finally melted at about 210°. Thin-layer chromatography of this 'Acipenserol-A' in ethyl formate gave a single spot with a mobility slightly greater than that of 5α-cyprinol. The infrared spectrum of 'Acipenserol-A' in KBr was indistinguishable from that of authentic 5β-cyprinol. This material (9mg.) was sent to Dr A. D. Cross for further examination (Cross *et al.* 1965). Material (5mg.) with the same properties as 'Acipenserol-A' was obtained by evaporation of later runnings (122–168ml.) of effluent.

In a similar manner, *A. guldenstaedti* and *A. stellatus* bile salts (2g. each) gave, after dioxan–trichloroacetic acid cleavage, 'bile alcohols' [0·12g. and 0·07g.; 6% and 3·5% (by wt.) respectively of bile salts]. Each bile alcohol fraction was left with a little ether and the insoluble portions were collected and washed with cold ether. Thin-layer chromatography in the solvent mixture benzene–propan-2-ol–acetic acid (30:10:1, by vol.) showed that the ether-insoluble solids gave spots with the R_F of 5β-cyprinol and also less polar spots, whereas the ether-soluble fractions contained small proportions of the less polar substances only. Ether-insoluble material from each species gave an infrared spectrum in KBr like that of 5β-cyprinol, but showing also 'allocholic acid nucleus' bands (Haslewood, 1967). Material of this type (49mg.) from *A. stellatus* was separated on Celite (10g.) in the system benzene–light petroleum (b.p. 80–100°)–ethanol–water (6:1:5:2, by vol.). Two main fractions (12 and 31mg., eluted by 16–44 and 50–100ml. respectively of moving phase) were collected. The less polar material (12mg.) was a complex mixture and the other fraction (31mg.) appeared spectroscopically and chromatographically to consist almost entirely of 5β-cyprinol.

Paddlefish bile salts

Bile of *P. spathula* Walbaum, preserved in ethanol, was obtained through the kindness of Miss J. Finstad and Dr J. B. Carey, jun., University of Minnesota Medical School, and also that of Dr E. A. Doisy, jun., St Louis University School of Medicine, and Mr T. Russell of the Missouri Conservation Commission. An 18ml. portion of bile was diluted with ethanol (excess) and the solution filtered. Evaporation left a gum that was extracted with methanol. The methanol was filtered and evaporated to give bile salts (1·44g.).

Alkaline hydrolysis. Bile salts (0·4g., prepared as described above) in water (3·2ml.) with 5N-NaOH (0·8ml.) were heated in a sealed metal bomb at 122° for 19hr. The diluted product contained a gelatinous solid, which was collected, washed with water and dissolved in methanol. Evaporation of the filtered solution left paddlefish 'alkaline-hydrolysis neutral materials' [8mg.; approx. 2% (by wt.) of bile salts] giving on chromatograms several spots, including one corresponding approximately to anhydro-5α-cyprinol and another running at about the same rate as 5α-cyprinol.

The alkaline filtrate from the 'alkaline-hydrolysis neutral materials' was treated with aqueous HCl and NaCl (excess) and refrigerated. Removal of the precipitated acids left a filtrate that with 0·5M-$BaCl_2$ (10ml.) gave $BaSO_4$ (17mg.). The acids were dissolved in ethanol–acetone and the filtered solution was evaporated. The residue was

extracted with hot ethyl acetate, which was filtered and evaporated, leaving paddlefish bile acids (201 mg.) as a solid. On paper chromatography, in the di-isopropyl ether–light petroleum–acetic acid–water system described above, a single spot corresponding to cholic acid was found. Esterification with ethanol gave ethyl esters (211 mg.), which after recrystallization from ether and then benzene gave a product (7 mg.) that had m.p. 188–190° and an infrared spectrum in KBr characteristic of ethyl cholate–ethyl allocholate mixtures previously examined (e.g. Anderson & Haslewood, 1962). No material less polar than ethyl cholate was detected on paper chromatography of material from the ether–benzene liquors. It was concluded that the bile acids consisted chiefly of cholic acid with a proportion (perhaps 1–2%) of allocholic acid.

Dioxan–trichloroacetic acid cleavage. Bile salts [0·2 g.; prepared as above, but in addition partially defatted by washing with cold light petroleum (b.p. 40–60°)] were acetylated and cleaved with 20% (w/w) dioxan–trichloroacetic acid reagent (15 ml.) as described by Haslewood (1964). The ether-soluble product was hydrolysed as described above for *H. huso* bile salts, giving finally paddlefish 'neutral materials' [11 mg.; approx. 5·5% (by wt.) of bile salts], which on thin-layer chromatography in the system chloroform–methanol–acetic acid–water (13:4:2:1, by vol.) gave a spot corresponding to 5β-cyprinol and at least three less polar spots.

Paddlefish 'neutral materials' (6 mg.) were separated on glass plates, with Kieselgel G (E. Merck A.-G., Darmstadt, Germany), thickness 0·25 mm., which were first washed by allowing methanol to run up to near the top of the plates. The plates were then dried at 110° and used for separation of paddlefish 'neutral materials' in the solvent mixture benzene–propan-2-ol–acetic acid (30:10:1, by vol.). Nearly contiguous spots, each containing 0·05 μg. of material in methanol, were placed on the start lines. After chromatography, plates were covered except for one or two lanes, which were sprayed with 10% (w/v) phosphomolybdic acid in ethanol. Heating at 100° revealed two main fractions: fraction I, consisting of a single spot with R_F about 0·2, and fraction II, two or three spots with R_F about 0·6. Unsprayed zones corresponding to the positions of fractions I and II were separately scraped off the plates and eluted with methanol. Evaporation of the filtered methanol left residues containing silica gel: this could be removed by washing with cold water. The water-insoluble material was largely dissolved by boiling with ethyl acetate. Evaporation of the filtered solvent left fraction I (2·0 mg.) and fraction II (1·6 mg.) as solids. Fraction I crystallized with a little ethyl acetate. The crystals (1·5 mg.) had m.p. 218–224°; the infrared spectrum of this material in KBr suggested that it was a mixture of 5α- and 5β-cyprinol. Fraction II was washed with ether. The ether-insoluble material gave an infrared spectrum in KBr showing bands characteristic of the cholic acid as well as of the allocholic acid nucleus (Haslewood, 1967).

New partial synthesis of 3α,7α,12α,26,27-pentahydroxy-5β-cholestane (5β-cyprinol)

Ethyl 3α,7α,12α-triacetoxy-5β-cholane-24-thiolate (III). Cholic acid (I) (80 g.) was dissolved in a mixture of acetic acid (640 ml.) and acetic anhydride (240 ml.). The solution was cooled to 10° and 60% (w/v) $HClO_4$ (0·5 ml.) was added dropwise. The solution was shaken between additions and cooled to maintain the temperature below 20°. After standing overnight at room temperature the solution was poured into water (3 l.), and the resulting suspension was extracted with ether (3 × 1 l.). The ether layers were combined, washed with water (5 × 1 l.), dried (Na_2SO_4) and evaporated to dryness, to yield triacetylcholic acid as a light-brown gum (105 g.). The gum was dissolved in dry benzene (1·2 l.) and some solvent (200 ml.) was removed by distillation to ensure dryness. Freshly distilled thionyl chloride (150 ml.) was added to the solution, which was then refluxed for 3 hr. with exclusion of moisture. The solvent and excess of thionyl chloride were removed by vacuum distillation with an N_2 leak on a steam bath. The residue was redissolved twice in benzene (400 ml.) and once in toluene (400 ml.); these solvents were removed similarly, to eliminate as much unchanged thionyl chloride as possible. The final residue was dissolved in benzene (500 ml.). Pyridine (50 ml.) and then ethanethiol (60 ml.) were added to this solution, which, with occasional shaking, was left for 40 hr. at 10–20°. A copious white precipitate formed. Ether (200 ml.) and water (200 ml.) were added, resulting in solution of the white precipitate in the aqueous layer. The aqueous layer was separated and extracted with ether (2 × 50 ml.). The combined organic phases were washed with aq. 1% (w/v) NaOH (2 × 200 ml.), 0·3 N-HCl (2 × 200 ml.) and water (2 × 200 ml.), dried (Na_2SO_4) and distilled *in vacuo* to dryness on a steam bath to yield a brown gum (151 g.), which was dissolved in benzene (300 ml.) and eluted from a column (1 kg.) of alumina (type H; Peter Spence and Sons Ltd., Widnes, Lancs.) as follows (fraction no., solvent, weight eluted): fraction 1, benzene (3 l.), 92 g., gum; fraction 2, benzene (2 l.), 2·5 g., gum. Fraction 1 was dissolved in methanol (270 ml.) and the solution filtered. Water (13 ml.) was added to the filtrate, which was kept overnight at 5°. The resulting crystalline mass was broken up, filtered at the pump, washed with cold aq. 95% (v/v) methanol and dried in a desiccator. The product (47 g.) was dissolved in methanol (130 ml.) and the solution filtered, treated with water (7 ml.) and cooled at 5° to yield white crystals of *ethyl 3α,7α,12α-triacetoxy-5β-cholane-24-thiolate* (III) (34 g.), m.p. 96–98° and $[\alpha]_D$ +73·4 ± 1° (*c* 2·7 in ethanol) (Found: C, 66·0; H, 8·3; S, 5·1; $C_{32}H_{50}O_7S$ requires C, 66·4; H, 8·7; S, 5·5%). Fraction 2 and the residue from the mother liquors of fraction 1 also crystallized with similar proportions of solvents and the total yield of recrystallized material was 56 g. (yield 49%, based on compound I).

3α,7α,12α-Triacetoxy-5β-cholan-24-ol (IV). The method used was an adaptation of that of Spero, McIntosh & Levin (1948). W–4 Raney nickel was made by the method of Pavlic & Adkins (1946) except that the nickel was washed 40 times by decantation instead of by using the procedure described. Ethyl 3α,7α,12α-triacetoxy-5β-cholane-24-thiolate (50 g.) was dissolved in ethanol (1 l.) and the solution added to W–4 Raney nickel made from Raney's alloy (500 g.). Water (250 ml.) was added to the mixture, which was then refluxed for 2 hr. The suspension was cooled and filtered at the pump. The nickel was washed with ethanol (2 × 250 ml.). The filtrate was evaporated to a total volume of about 1 l., cooled, poured into a separating funnel and there diluted with water (2 l.). The resulting white suspension was extracted with ether (3 × 1 l.). The ether extracts were combined, washed with aq. 1% (w/v)

NaOH (2 × 1 l.) and water (3 × 1 l.), dried (Na_2SO_4) and evaporated to dryness under reduced pressure to yield a colourless gum (42 g.). This was dissolved in ether (400 ml.); on standing, a white crystalline mass formed that was broken up, collected at the pump, washed with ether and recrystallized from ether to yield white crystals of *3α,7α,12α-triacetoxy-5β-cholan-24-ol* (IV) (27 g.; yield 60%, based on compound III), m.p. 150–152° and $[\alpha]_D$ +82·6 ± 1° (c 2·4 in ethanol) (Found: C, 69·8; H, 9·3; $C_{30}H_{48}O_7$ requires C, 69·2; H, 9·3%).

Diethyl 3α,7α,12α-triacetoxy-5β-cholestane-26,27-dicarboxylate (VI). Triphenyl phosphite methiodide was prepared by the method of Landauer & Rydon (1953); no attempt was made to purify the crude product. To triphenyl phosphite methiodide (8·0 g.) in a dry flask was added 3α,7α,12α-triacetoxy-5β-cholan-24-ol (8·0 g.) and methyl iodide (20 ml.). The mixture was refluxed with the exclusion of moisture for 1 hr. and left overnight at room temperature. Light petroleum (b.p. 40–60°) (100 ml.) was added and the contents of the flask were thoroughly shaken. On standing, an apparently insoluble dark-brown gum formed at the bottom of the flask. The supernatant petroleum layer was decanted into a separating funnel and washed with aq. 10% (w/v) $Na_2S_2O_3$ (50 ml.), 0·1 N-NaOH (5 × 100 ml.) and water (2 × 100 ml.), dried (Na_2SO_4) and evaporated to dryness to yield a colourless gum (*A*), presumably largely compound (V).

Sodium (1 g.) in boiling anhydrous toluene (100 ml.) was stirred vigorously under reflux to produce a fine suspension. Diethyl malonate (8 ml.) was added; after 1 hr. all the sodium had apparently reacted to form diethyl sodiomalonate. The solution was cooled to room temperature and to it was added a solution in toluene (20 ml.) of the gum *A*. The mixture was heated gently, with stirring, and at about 85° a precipitate began to form. The heat was increased to cause gentle refluxing of the toluene, which was continued for 5 hr. The solution was cooled and filtered. The filtrate was washed with aq. 10% (w/v) $Na_2S_2O_3$ (50 ml.), 0·1 N-NaOH (2 × 100 ml.) and water (2 × 200 ml.), dried (Na_2SO_4) and evaporated to dryness under reduced pressure with an air leak, to yield a light-yellow viscous fluid. This was dissolved in methanol (20 ml.), and water (2 ml.) was added. After 30 min. at 0° the solution deposited a mass of crystals. These were filtered off and recrystallized from aq. 90% (v/v) methanol (20 ml.) to yield *diethyl 3α,7α,12α-triacetoxy-5β-cholestane-26,27-dicarboxylate* (VI) (4·2 g.; yield 41%, based on compound IV), m.p. 142–145° and $[\alpha]_D$ +65·6 ± 1° (c 2·2 in ethanol) (Found: C, 67·0; H, 8·6; $C_{37}H_{58}O_{10}$ requires C, 67·0; H, 8·8%).

3α,7α,12α,26,27 - Pentahydroxy - 5β - cholestane (VII). Diethyl 3α,7α,12α - triacetoxy - 5β - cholestane - 26,27 - dicarboxylate (2·0 g.) was dissolved in dry tetrahydrofuran (50 ml.). $LiAlH_4$ (1·0 g.) was added to the solution in portions during 30 min., with cooling in ice–water between additions. The resulting suspension was shaken at room temperature for 15 min. and then refluxed for 45 min. After cooling, the suspension was poured into ice-cold 2 N-H_2SO_4 (300 ml.). This mixture was extracted with ethyl acetate (3 × 100 ml.) and the combined extracts were washed with saturated $NaHCO_3$ (100 ml.) and water (3 × 100 ml.), dried (Na_2SO_4) and evaporated almost to dryness to yield a white crystalline substance (1·40 g.). The crystals were twice recrystallized from ethyl acetate to yield material (860 mg.) almost free of less polar impurity. The mother liquors were combined to yield, on evaporation, a gum (500 mg.) that was purified on a column of neutralized (Evans & Shoppee, 1953) alumina (15 g.). Thin-layer chromatography of the column fractions showed that four fractions [total weight 188 mg.; eluted by 10% (v/v) methanol in ethyl acetate] consisted of almost pure 5β-cyprinol. These were combined and recrystallized from ethyl acetate to yield 3α,7α,12α,26,27-pentahydroxy-5β-cholestane (5β-cyprinol, VII) as crystals (103 mg.; yield 7% based on compound VI), m.p. 175–177° and $[\alpha]_D$ +35·5 ± 1° (c 1·7 in ethanol) (Found: C, 71·4; H, 10·8. Calc. for $C_{27}H_{48}O_5$: C, 71·6; H, 10·7%). Hoshita *et al.* (1963) give m.p. 173–174° for this compound.

Dr A. D. Cross reported that n.m.r. spectra of this substance (measured at 60 Mcyc./sec. in heptadeuterodimethylformamide with and without added D_2O) were consistent with its identity as compound (VII) and as 'Acipenserol-A' (Cross *et al.* 1965). The infrared spectrum of the partially synthetic 5β-cyprinol in KBr was indistinguishable from that of 'Acipenserol-A' and from that of the substance, m.p. 171–173°, made by Bridgwater *et al.* (1962).

DISCUSSION

Biological. The most primitive extant osteichtheans (*Latimeria*, Dipnoi) have a mixture of C_{27} bile alcohol sulphates as the chief or sole functional bile salts. Bile acids have not been isolated from these forms and all the bile alcohols identified (which include 5α-cyprinol in every case) have the 5α-configuration. On the other hand, 5β-cyprinol has been found (Sasaki, 1966) in traces in certain eels (Apodes), and alcohol sulphates of the 5β-configuration constitute the chief bile salts of chondrichthean fishes (selachians).

If bile salt evolution in osteichtheans proceeded from alcohol sulphates to taurine conjugates of allocholic acid and cholic acid, as seems likely, then the chondrostean bile salts now described can be considered to be mainly advanced in their chemistry, but with clearly primitive features; thus in their general nature they agree with Romer's (1966) assessment of the chondrostean phenotype. The fact that sturgeon and paddlefish bile salts, though of an unusual kind, closely resemble one another is likewise in accordance with the taxonomic affinities of these fishes.

If any evolutionary significance can be attached to the chemical nature of sturgeon and paddlefish bile alcohols, a doubtful question discussed below, the presence of 5α- and 5β-cyprinol agrees with chondrostean descent from ancestors common to Chondrostei and early Osteichthyes. However, the chemical nature (high proportions both of C_{27} pentols and of substances behaving like C_{27} tetrols) of the (presumably) relict alcohols in sturgeon and paddlefish bile is such as to suggest that, if such a mixture constituted (as its sulphates) the principal bile salts, it could, by analogy with bile salts in other vertebrate forms, hardly function effectively in a

physiological sense. In other words, the chondrostean bile alcohols seem to make no physiological 'sense'. An explanation of this situation might be that, as taurine conjugates of bile acids took over the functions of bile salts, selection pressure was lifted from the alcohol sulphates, which then (perhaps by accumulation of unfavourable mutations in the DNA ultimately responsible for their biosynthesis) became functionally useless, much as (for example) in the case of limb rudiments in primitive snakes. If this was indeed the history of these bile alcohols, it will probably be fruitless, in our present state of understanding of chemical evolution, to inquire too closely into the possible evolutionary significance of single constituents of the bile alcohol mixture.

Allocholic acid, in small proportions, is found in the bile of a number of teleostean fish; it probably indicates that the remote ancestors of such animals had bile alcohols of the 5α-configuration. Its presence in chondrostean bile seems to add nothing to the biological deductions set out above.

In summary, then, the chemical nature of sturgeon and paddlefish bile salts supports the accepted systematic view of the history and relationships of these fishes and also shows features that suggest yet another similarity between the nature of evolution as observed at molecular and at phenotypic levels. Repeated coincidence of evolutionary pictures elucidated in widely different degrees of detail is an encouraging feature of comparative studies of bile salts.

Chemical. There seems little doubt that the chief constituent of 'Acipenserol-A' is 5β-cyprinol; the difference in properties (m.p., crystalline form) might be explained by the presence of a little 5α-cyprinol, apparently present in greater proportion in paddlefish bile alcohols.

We cannot account for our failure to make 5β-cyprinol by the method of Hoshita *et al.* (1963). In our hands the lithium aluminium hydride reduction of the product of the reaction between 2,3-dihydropyran and methyl $7\alpha,12\alpha$-diacetoxy-3α-hydroxy-5β-cholanoate afforded mainly $3\alpha,7\alpha,12\alpha,24$-tetrahydroxy-$5\beta$-cholane, together with a small amount of a less polar gum. The product obtained by treating this gum with toluene-*p*-sulphonyl chloride failed to condense with diethyl sodiomalonate. Our new partial synthesis hardly leaves room for doubt as to the correctness of formula (VII) for the final product.

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