Comparative Studies of Bile Salts

MYXINOL DISULPHATE, THE PRINCIPAL BILE SALT OF HAGFISH (*MYXINIDAE*)

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1. The principal bile salt, myxinol disulphate, of two hagfish species, *Eptatretus stoutii* and *Myxine glutinosa*, has been shown by chemical methods and by optical-rotatory and mass-spectral considerations to be probably the C-3,27-disulphate ester of 3β,7α,16α,27-tetrahydroxy-5β-cholestane. 2. *Myxine* liver sterols were about half 'free' and half esterified: cholesterol only was identified. 3. The chemical nature of myxinol is what might be expected for the bile alcohol of a very primitive vertebrate.

The most primitive craniate vertebrates, the hagfishes (Family Myxinidae), have very large gall bladders. The bile salts prepared in the usual way from two species were found to consist chiefly of a disulphate ester, crystallizing as the disodium salt (Haslewood, 1961*a*).

The parent alcohol, myxinol, has now been derived from its sulphate, and its chemistry, and that of the natural sulphate, elucidated; a preliminary note of this work has appeared (Cross & Haslewood, 1965).

RESULTS

Bile salts of the Atlantic *Myxine glutinosa* and the Californian species, *Eptatretus stoutii*, readily yielded the same disulphate, already described as the sodium and barium salts (Haslewood, 1961*a*).

The sulphate was acetylated and treated with dioxan–trichloroacetic acid reagent: hydrolysis of the ether-soluble neutral product readily gave a crystalline alcohol, myxinol, which after purification had m.p. 206° and $[\alpha]_D$ −15°, and had an infrared spectrum different from that given by any bile acid or alcohol examined in this Laboratory; a particularly striking feature was a strong band at about 11·4μ (Fig. 1). Myxinol with cold acetic anhydride–pyridine or by the perchloric acid method gave a tetra-acetate, m.p. 198° and $[\alpha]_D$ −60°, recovered unchanged after treatment with hydrogen and platinum catalyst, the analysis of which agreed with the formula $C_{27}H_{48}O_4$ for myxinol.

A preliminary mass-spectral examination of myxinol tetra-acetate showed strong peaks at *m/e* 253 and 409: the former in other bile acids and alcohols is attributed to the ion $C_{19}K_{25}^+$, arising by elimination of the side chain and of three molecules of acetic acid or water from the tetracyclic nucleus. The peak at *m/e* 409 can be assigned to an ion (*M*-195) formed by the elimination of three molecules of acetic acid and a methyl group from an acetate of mol.wt. (*M*) 604. Thus this evidence supported the formula $C_{27}H_{48}O_4$ for myxinol, the molecule of which is saturated and has four hydroxyl groups, all easily acetylated. Myxinol reacted only slightly with lead tetra-acetate; hence there are probably no vicinal hydroxyl groups.

Cold chromic acid oxidation converted myxinol into an acid (II), titration of which agreed with a formula $C_{27}H_{40}O_5$ (one $-CO_2H$ group); hence only one $-CH_2\cdot OH$ group is present in myxinol.

Myxinol formed a digitonide that could be obtained by dilution of an ethanolic solution with water, suggesting that a 3β-hydroxyl group is present, probably in a coprostane (5β-H) nucleus. If it is assumed that the $-CH_2\cdot OH$ group is at the end of the side chain, the remaining two hydroxyl groups, which must be in the ring nucleus, can be assigned as follows. No bile acid or alcohol that

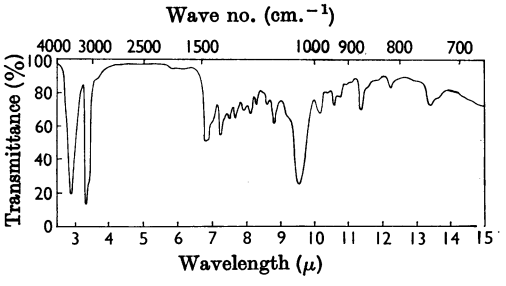
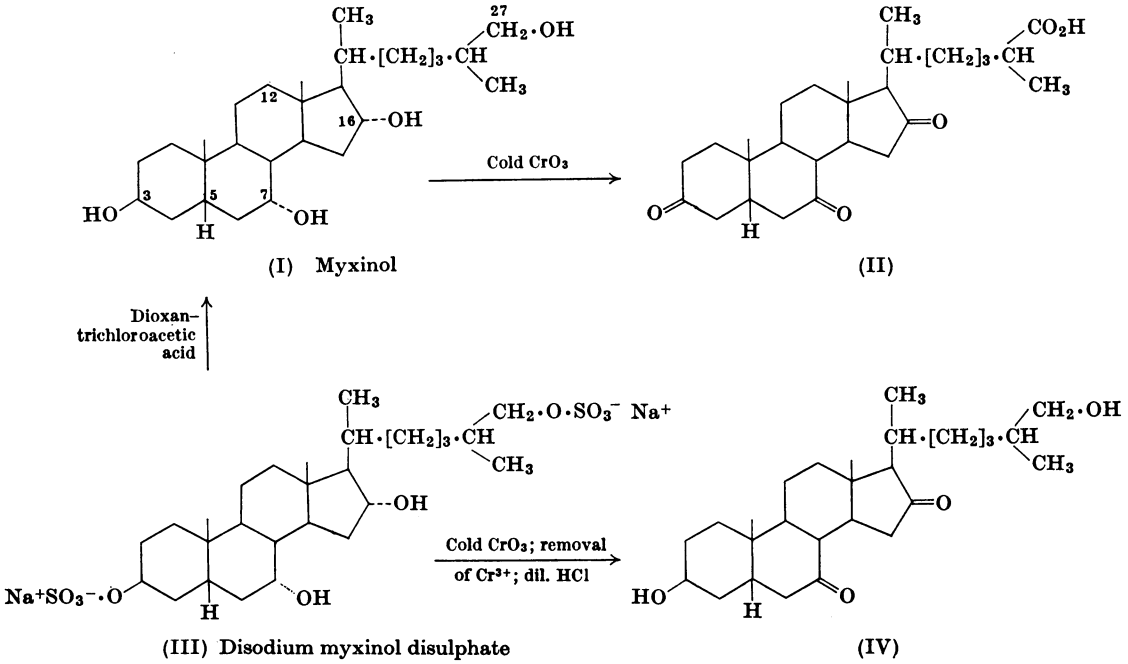


Fig. 1. Infrared spectrum of myxinol in KBr.



(I) Myxinol (II)

(III) Disodium myxinol disulphate (IV)

is 'primary' (i.e. made by the liver from cholesterol) is known that does not contain a hydroxyl group at C-7; if this is assumed for myxinol, M_D values indicate that the remaining group must be at C-16α and can in fact be reconciled only with hydroxyl groups at C-7α and C-16α (see the Experimental section).

Hence myxinol can be given the structure 3β,7α,-16α,27-tetrahydroxy-5β-cholestane (I). Nuclear-magnetic-resonance and detailed mass-spectral examinations led Dr A. D. Cross independently and simultaneously to the same conclusion (see the Appendix).

Myxinol sulphate was oxidized with cold chromic acid, Cr^{3+} was removed, and the organic product hydrolysed with aq. hydrochloric acid. A crystalline neutral substance resulted, which readily formed a digitonide. This neutral substance, characterized as a diacetate, must therefore have structure (IV), which is supported by consideration of its M_D, and hence the natural bile salt, sodium myxinol disulphate, has formula (III).

Both the crude bile salts and the products of dioxan–trichloroacetic acid cleavage of these showed on paper chromatograms spots corresponding to minor amounts of a substance less polar than myxinol disulphate or myxinol, suggesting that at least one other compound, as its sulphate, is present in hagfish bile salts.

The digitonin-precipitable sterols, naturally free and esterified, of *Myxine glutinosa* liver and bile were isolated: there was no gas–liquid-chromatographic evidence that they contained any sterol other than cholesterol.

EXPERIMENTAL

Materials and methods

These were as described by Bridgwater, Haslewood & Watt (1963).

Hagfish bile salts

Gall bladders of *Eptatretus stoutii* or of *Myxine glutinosa* were cut under ethanol (excess) and the solutions filtered. Evaporation left the bile salts (for example, 70 specimens of *E. stoutii* gave 2·23g.; 14 specimens not fed for 10 months gave 1·79g.; 24 specimens of *M. glutinosa* gave 0·88g. of light-brown solid bile salts: those from the starved hagfish contained much apparently inorganic material). Chromatography on paper in the solvent system pentyl acetate–heptane–acetic acid–water (17:3:14:6, by vol.) (Sjövall, 1955) showed that the bile salts contained at least one major and one minor component, both more polar than taurocholate. Evaporation of the original filtered ethanolic solution of the bile, or treatment of the crude bile salts with fresh ethanol, caused the separation of a crystalline solid. This could be purified by warming with methanol, followed by filtration from the mainly inorganic undissolved material. Evaporation of the methanol left purified bile salts, mainly *sodium myxinol disulphate*, as crystals, which could be further purified by recrystallization from methanol or ethanol. The purest specimens had a very bitter taste, showed a single spot on paper chromatograms and had

m.p. approx. 195° (decomp.) [Found: Na^+, 6·8; S (after alkaline hydrolysis as described below), 8·0. $C_{27}H_{46}O_{10}S_2Na_2$ requires Na, 6·9; S, 10·0%]. The purified salt (13·3mg.) in water (0·5ml.) was treated with 0·5M-$BaCl_2$ solution (1ml.). The crystals that separated were collected, washed with cold water and dried *in vacuo* over $CaCl_2$. The yield was 11·9mg. of *barium myxinol disulphate*, m.p. 147–148° (decomp.) (Found: C, 42·1; H, 6·5; Ba^{2+}, 17·5. $C_{27}H_{46}O_{10}S_2Ba,2H_2O$ requires C, 42·2; H, 6·3; Ba, 17·9%). In the solvent system (S_1) pentyl acetate–heptane–acetic acid–water (17:3:21:10, by vol.), the barium salt gave a single spot with an R_F much less than that of any other bile alcohol sulphate.

Cleavage of myxinol disulphate

Alkaline hydrolysis. The purified bile salts (50mg.) were added in a metal bomb to a solution made by dissolving sodium (0·1g.) in ethanol (1ml.) and water (1ml.). Hydrazine hydrate (0·2ml. of '90/100%') was added and the bomb sealed and heated at 181±1° for 19hr. The precipitated product was a neutral white solid (31·4mg.). (The acidified liquors, with 5ml. of 0·5M-$BaCl_2$, gave 33·0mg. of $BaSO_4$.) An experiment without hydrolysis gave 3·7mg. of $BaSO_4$: hence SO_4^{2-} liberated on hydrolysis corresponded to S, 8·0%. The neutral solid did not crystallize. Bile salts (200mg.) in 2N-NaOH (10ml.) in a sealed metal bomb were heated at 158±1° for 18hr. Hydrolysis was partial: the neutral material weighed 21mg. and from ethyl acetate gave small white crystals, m.p. 140–143°, giving a single spot on paper chromatography in system G_3 (Haslewood & Sjövall, 1954) and an infrared spectrum similar to that of myxinol (see below).

Acid hydrolysis. Purified bile salts (50mg.) in 0·25N-HCl (2ml.) were heated in a boiling-water bath under reflux for 19hr. Partial hydrolysis occurred: the precipitated neutral material (10mg.) from ethyl acetate gave globules of m.p. approx. 198°, whose infrared spectrum was also similar to that given by myxinol and which on paper chromatography in system G_3 gave a single spot less polar than that given by the alkaline-hydrolysis product (above).

Dioxan–trichloroacetic acid cleavage. Purified bile salts (0·4g.) in acetic acid (8ml.) and acetic anhydride (8ml.) were heated on a boiling-water bath under reflux for 1hr. Solvent was removed, finally *in vacuo*, and the dry residue treated immediately with a 40% (w/w) solution (8ml.) of freshly distilled trichloroacetic acid in dry dioxan. The mixture was left in the dark with occasional shaking for 20 days: it was then diluted with water and extracted thrice with ether. The ether was washed with water, aq. NH_3 and water, dried over Na_2SO_4 and evaporated. The residue was heated for 30min. under reflux with ethanol (10ml.) and 5N-NaOH (2ml.). Solvent was removed with N_2 and the residue diluted with water; the solid product was collected on a filter and well washed. After being dried, this material (198mg.) was dissolved in acetic acid (4ml.) with acetic anhydride (0·8ml.) and the solution left for 15min. after the addition of 8·5N-$HClO_4$ (0·05ml.). Water was then added and the product recovered by three extractions with ether. The ether, washed and dried as above, was evaporated, leaving a residue that from benzene–light petroleum gave *myxinol tetra-acetate*, a recrystallized sample of which formed white leaflets of m.p. 196–198° and $[\alpha]_D - 60 \pm 1°$ (*c* 1·0 in $CHCl_3$) (Found: C, 69·8; H, 9·6. $C_{35}H_{56}O_8$ requires C, 69·5; H, 9·3%). The same substance was formed by acetylation overnight of myxinol with acetic anhydride–pyridine (1:1, v/v) at room temperature. This acetate (18mg.) was recovered unchanged (m.p., mixed m.p. and infrared spectrum) after shaking for 2hr. in ethanol (2ml.) with 10N-H_2SO_4 (0·4ml.) and Adams platinum catalyst (20mg.) (experiment carried out by Dr I. G. Anderson). Myxinol tetra-acetate (190mg.) was heated for 15min. under reflux with ethanol (10ml.) and 5N-NaOH (1ml.). Solvent was removed in N_2 and the solid residue collected and washed with water. The product could be crystallized from wet ethyl acetate to give colourless glistening (solvated) leaflets (140mg.) of *myxinol*, m.p. 204–206°, $[\alpha]_D - 15 \pm 2°$ (*c* 1·2 in ethanol) and infrared spectrum as shown in Fig. 1 (Found, on a purified sample dried to constant weight at 80° *in vacuo*: C, 71·4; H, 10·9. $C_{27}H_{48}O_4$ requires C, 71·7; H, 10·6%). Myxinol dissolved readily in methanol and ethanol; it was less soluble in chloroform, ether, benzene and acetone. On paper chromatograms in system G_3, it had an R_F about twice that of 5α-cyprinol: crude preparations gave a faint spot running just ahead of that due to myxinol itself and another near the solvent front. (The author and Professor T. Kazuno have agreed to use the trivial names bufol, cyprinol and ranol for 3α,7α,12α,25ξ,27- and 3α,7α,12α,26,27-pentahydroxycholestane and 3α,7α12α,24ξ,26-pentahydroxy-27-norcholestane respectively, with the prefixes 5α- or 5β- to indicate the configuration at C-5. Thus what have been previously called cyprinol and ranol are now 5α-cyprinol and 5α-ranol; 'bufol' is a newly introduced name.) Myxinol, like its disulphate and tetra-acetate, gave an intense maroon–crimson colour in the Liebermann–Burchardt reaction; a similar colour was given by pythocholic (3α,12α,16α-trihydroxycholanic) acid and was much more intense than that given by cholic acid, deoxycholic acid, chenodeoxycholic acid or 3β,7α-dihydroxycholanic acid. It seems probable that the 16α-hydroxyl group is responsible for the augmented response of pythocholic acid and myxinol.

Myxinol digitonide. Myxinol (6mg.) was dissolved by warming with 2·5ml. of a solution (1%, w/v) of digitonin in ethanol–water (2:1, v/v). Water was added until the ethanol–water ratio was 1:2 (v/v). After 4 days, the gelatinous precipitate was collected, washed with aq. 30% (v/v) ethanol and dried *in vacuo* over $CaCl_2$. The yield was 8mg. of material obviously not myxinol. This substance was decomposed with pyridine and ether in the usual way, giving myxinol (3mg.), identified by m.p., mixed m.p. and infrared spectrum, and digitonin, identified by precipitation with an ethanolic solution of cholesterol.

In the conditions previously used (Haslewood, 1961*b*) myxinol reacted with lead tetra-acetate to consume about 0·2 atom-equiv. of oxygen; phocaecholic (3α,7α,23ξ-trihydroxycholanic) acid and hyocholic (3α,6α,7α-trihydroxycholanic) acid each consumed about 1 atom-equiv. of oxygen, and 5α-ranol and 5α-cyprinol were unreactive. The small but definite reaction with myxinol might be attributed to the 16α-hydroxyl group.

Chromic acid oxidation of myxinol

Myxinol (140mg.) in acetic acid (3ml.) was treated with 20% CrO_3 (1·4ml.) added gradually from a burette, with frequent mixing. The temperature rose to about 35°,

and the mixture was allowed to stand at about 30° for 2hr. Water and NaCl (excess) were added and the product was refrigerated overnight. The precipitate was collected, washed with water and stirred with warm aq. $NaHCO_3$. The filtered solution was treated with 2N-HCl and NaCl (excess) and the precipitated acid extracted with ether. The ether was washed with water, dried over Na_2SO_4 and evaporated, leaving a colourless gum (104mg.) that on titration had equiv.wt. 448. With a little ether, large colourless crystals (43mg.) formed: these were recrystallized from aq. ethanol to give short white (solvated) needles (37mg.) of (presumably) 3,7,16-*trioxocoprostanic acid* (II), which appeared to lose solvent at about 150°, then recrystallized and melted at about 160°, $[\alpha]_D - 124 \pm 1°$ (*c* 0·95 in ethanol) [Found, C, 72·0; H, 9·15. $C_{27}H_{40}O_5$ requires C, 73·0; H, 9·0%; equiv.wt. (one $-CO_2H$ group), 444]. Attempts to convert this substance into coprostanic acid by Kishner–Wolff reduction proved abortive: the final product was contaminated, apparently with hydrocarbon.

Chromic acid oxidation of myxinol disulphate

The disulphate (0·2g.) in acetic acid (2ml.) was treated at room temperature with 20% CrO_3 (0·6ml.) added gradually, with mixing, from a burette. After 2hr., methanol (2ml.) was added and the mixture left overnight. The Cr^{3+} was removed by steam-distillation and warming with NaOH, as previously described (Haslewood, 1964), the colourless final filtrate was evaporated and the residue, in 0·25N-HCl (8ml.), heated under reflux for 28hr. NaCl (excess) was added and, after refrigeration, the precipitated solid was collected, washed with water and extracted with a mixture of methanol and acetone. The filtered extract was evaporated and the residue (90mg.) crystallized with ether and then aq. ethanol to give colourless needles (54mg.), which lost solvent after heating at 100° and then had m.p. 163–169°. This substance, presumably 3β,27-*dihydroxy*-7,16-*dioxo*-5β-*cholestane* (IV), had $[\alpha]_D - 149 \pm 1°$ (*c* 0·9 in ethanol) (Found: C, 74·3; H, 10·0. $C_{27}H_{44}O_4$ requires C, 75·0; H, 10·2%). This compound was insoluble in dilute aq. alkali. It (1mg.) was dissolved by warming with 0·5ml. of a 1% (w/v) solution of digitonin in ethanol–water (2:1, v/v): a thick precipitate appeared on cooling.

The compound (IV) was acetylated as described for myxinol. The product, from light petroleum, formed colourless needles of 3β,27-*diacetoxy*-7,16-*dioxo*-5β-*cholestane*, m.p. 134–137° [Found: C, 72·0; H, 9·55. $C_{31}H_{48}O_6$ (diacetate of IV) requires C, 72·1; H, 9·3%].

Sterols and fatty acids from Myxine glutinosa *liver*

Livers from 30 fish were kept in ethanol: for working up, the livers were extracted in a Soxhlet apparatus with ether and the extract was combined with the residue from the evaporation of the ethanol. The total ethereal extract was washed with water, aq. NH_3 and water, dried over Na_2SO_4 and evaporated, leaving an orange oil (4·77g.), which was mixed with aq. 90% (v/v) ethanol (20ml.) and a solution of digitonin (1g.) in aq. 90% (v/v) ethanol (100ml.). The precipitated ('free sterol') digitonide weighed 0·42g., and no further precipitate was formed on treatment with digitonin of the oil (4·48g.) recovered from the digitonide liquors. Part of this recovered oil (1·89g.) was heated under reflux for 70min. in ethanol (20ml.) in a slow current of N_2 after the addition of 5N-NaOH (2ml.). The ether-soluble unsaponified material, recovered in the usual way, was an orange gum (0·20g.) and the ether-soluble acids (1·24g.) formed a partly crystalline yellow oil. The unsaponifiable material (0·20g.) gave an ('esterified sterol') digitonide (0·18g.), prepared in 90% (v/v) ethanol as described above. Thus from the total liver fat the weights of digitonides were: 'free sterols', 0·42g.; 'esterified sterols', $0·18 \times 4·48/1·89 = 0·43$g., and hence about half the digitonin-precipitable sterols were 'free' in the liver fat. Decomposition of the digitonides with pyridine–ether in the usual way gave crystalline sterols, which on gas–liquid chromatography (by Dr D. B. Gower) appeared to consist almost entirely of cholesterol. Similar chromatography (by Dr I. Macdonald) of the isolated liver fatty acids (1·24g., above) gave the following analysis (percentage by wt.): $C_{14:0}$, 8·4; $C_{16:0}$, 17·2; $C_{16:1}$, 18·3; $C_{18:1}$, 47·6; $C_{20:1}$, 8·5 (where $C_{x:y}$ represents a fatty acid with x carbon atoms and y double bonds/molecule).

Considerations of M_D

To obtain a value for C-16α-acetoxy in the 5β-cholane system, methyl pythocholate (methyl 3α,12α,16α-trihydroxycholanate) (27mg.) was acetylated as described

Table 1. *Calculated and found values for* M_D *for myxinol and its tetra-acetate*

M_D increments were taken from Fieser & Fieser (1959), except where indicated.

	Myxinol		Myxinol tetra-acetate
M_D increment for:		M_D increment for:	
5β-Cholestane	+93°	5β-Cholestane	+ 93°
3β-OH	+ 1	3β-O·CO·CH_3	+ 18
7α-OH	−79	7α-O·CO·CH_3	−144
16α-OH*	−72	16α-O·CO·CH_3†	−381
Calculated M_D	−57	Calculated M_D	−414
Found M_D	−65	Found M_D	−362

* From Haslewood & Wootton (1951).
† See the text.

for myxinol, and the (non-crystalline) product purified by elution from neutralized alumina with benzene. The eluted colourless gum (28mg.) had $[\alpha]_D + 15 \pm 1°$ (*c* 1·9 in ethanol); hence $M_D = +82°$. Koechlin & Reichstein (1942) give $[\alpha]_D + 94·4°$ ($M_D + 463°$) for methyl diacetyldeoxycholate (methyl 3α,12α-diacetoxycholanate). Hence M_D for 16α-acetoxy = 82° − 463° = −381°. This value was used in compiling Table 1. The M_D values for substances (II) and (IV) are also in reasonable agreement with the calculated values.

DISCUSSION

Chemical. The structure (I) for myxinol is proposed on the basis of analytical values, chemical behaviour and physical measurements (mass spectra, nuclear magnetic resonance, M_D). Attempts to convert myxinol into a substance (coprostanic acid) already known have not been convincingly successful. Nevertheless, the formula (I) can be regarded as well supported by the available evidence, although it ought to be further tested by reference to compounds of known structure.

Biological. The Myxinidae have been called the most primitive craniate chordates known and represent the specialized descendants of very ancient vertebrate stock (Heintz, 1963). They might therefore be expected to have bile salts of an evolutionarily primitive type, perhaps early examples of the biogenesis of such substances from sterols. The formula of myxinol disulphate (III) accords well with such an idea. The hydroxyl group at C-3 is in the β-position, as in cholesterol; the only other example of this in a bile alcohol is in latimerol (from the coelacanth, *Latimeria chalumnae*), which it was suggested might be elaborated by an animal too primitive to possess the full complement of enzymes necessary to bring about inversion at C-3 (Anderson & Haslewood, 1964).

Hydroxylation at C-16α might have been an early step in bile alcohol biosynthesis, before hydroxylation at C-12α, for every other vertebrate examined has been shown to be able to hydroxylate at C-12α or to be closely related to animals having this ability. The only other animals known to be capable of 16α-hydroxylation are certain primitive snakes, and in their case the reaction appears to be a response to the formation of deoxycholic acid from cholic acid during the enterohepatic circulation.

Myxinol is the only tetrol so far discovered certainly acting as a functional bile alcohol; other such alcohols have at least five hydroxyl groups, and it is tempting to suggest that it was necessary for a disulphate (the only example known amongst bile alcohol sulphates) to be formed to provide a substance with (? detergent) properties sufficiently marked to function effectively as a bile salt. In model, the myxinol disulphate molecule does not, in contrast with those of other bile alcohol sulphates and of taurocholate, look particularly like an efficient detergent, for the hydrophilic and lipophilic parts of the molecule are not sharply separated. One may speculate that this is one reason why the Myxinidae require such relatively enormous quantities of their peculiar bile salt.

The fact that myxinol has the C-5β configuration suggests that this is an early state of affairs. No C-5α (allo) bile salts have as yet been found among the primarily marine selachians and perhaps allo bile salts arose in freshwater vertebrate forms and are now an indication of an ancestry including such forms. This idea can be tested by further comparison between the chemistry of bile salts and other indicators of phylogeny; at present it is highly speculative.

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