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Comparative Studies of Bile Salts

BILE SALTS OF THE LAMPREY *PETROMYZON MARINUS* L.

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1. Bile salts of *Petromyzon marinus* L. ammocoetes appeared to consist solely or chiefly of a crystalline substance, whose chromatographic and i.r.-spectral characteristics suggested that it was a monosulphate ester of a bile alcohol having the 3α,7α,12α-trihydroxy pattern of substitution in a 5α-steroid nucleus. 2. This substance on cleavage with dioxan–trichloroacetic acid gave petromyzonol, n.m.r. and mass-spectral examination of which suggested the structure 5α-cholane-3α,7α,12α,24-tetrol. 3. 3α,7α,12α-Trihydroxy-5α-cholanoic acid (allocholic acid) from the lizards *Anolis lineatopus lineatopus* Gray and *Cyclura carinata* Harlan (family Iguanidae) was esterified with propan-1-ol and reduced by lithium aluminium hydride to 5α-cholane-3α,7α,12α,24-tetrol, identical with petromyzonol. 4. Chromic acid oxidation of petromyzonol sulphate from lamprey bile, followed by acid hydrolysis, gave 24-hydroxy-5α-cholane-3,7,12-trione; hence the sulphate ester group is at C-24. 5. Petromyzonol sulphate is both primitive and unique: a study of its biogenesis might improve our understanding of evolution at the molecular level.

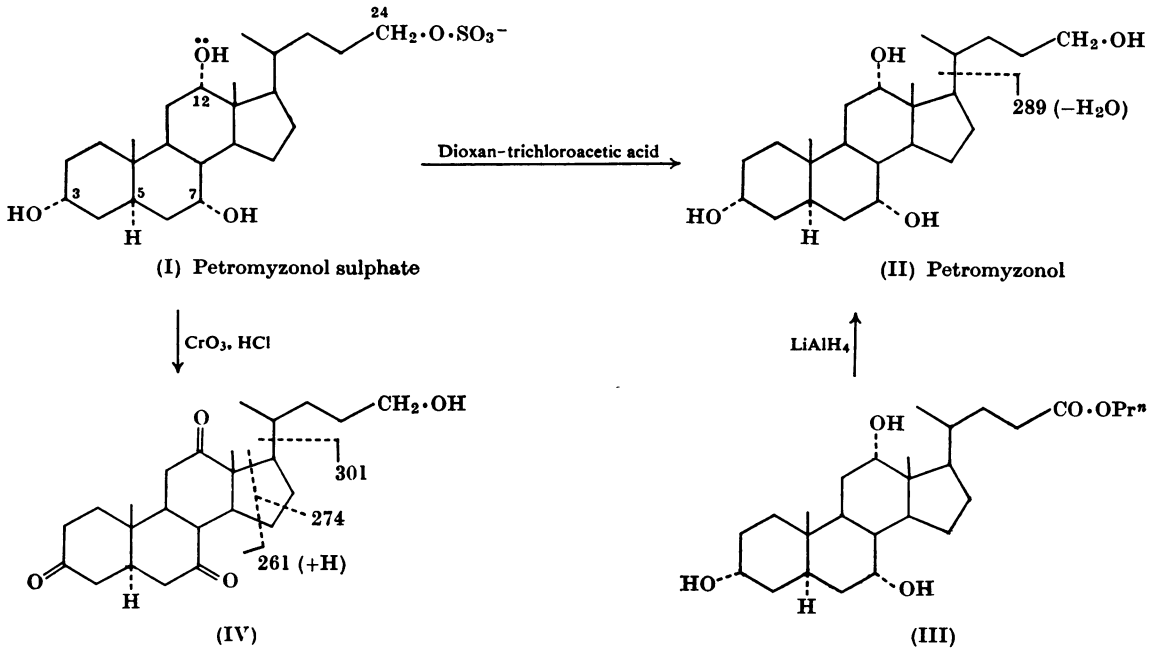
Lampreys and hagfishes, the sole survivors of the jawless fishes (Agnatha), are of outstanding interest to students of vertebrate evolution. Highly specialized as these forms now are, it nevertheless seems likely that a knowledge of some of their biochemical characters will throw light on primitive mechanisms that must have played an important part in determining the course of later evolution.

We have described studies of hagfish bile salts (Anderson, Haslewood, Cross & Tökés, 1967; Anderson & Haslewood, 1969; Tökés, 1969) and now report on those of the lamprey *Petromyzon marinus* L. In adult lampreys, parasitic on other fishes, the digestive system is partly degenerate and we have not been able to obtain bile from these; however the ammocoete (larval form) is free-living and has a gall-bladder. Through the kindness of other workers we obtained sufficient bile from such animals for our study, which we summarized in a preliminary communication (Haslewood & Tökés, 1968).

RESULTS

Earlier small samples of lamprey bile salts (including one possibly from *Petromyzon flaviatilis* Day) gave on paper chromatography a single spot with a mobility greater than that of taurocholate and much greater than that of hagfish bile salts. *P. marinus* bile salts gave a positive Hammarsten (hydrochloric acid) test and an i.r. spectrum that had the principal bands at about 9·2, 9·7, 9·9, 10·4 and 11·2 μm. attributed to the allocholic acid ring nucleus (Haslewood, 1967*a*). The spectrum differed somewhat from that of the bile salts of the grass carp *Ctenopharyngodon idella*, which we believe to consist chiefly of 5α-cyprinol sulphate. These results were interpreted as meaning that *P. marinus* bile salts consisted largely or entirely of the monosulphate of a previously unknown steroid alcohol having the 5α-configuration and a 3α,7α,-12α-pattern of hydroxyl substitution (Haslewood, 1967*b*).

A larger sample of *P. marinus* bile easily yielded a crystalline sulphate, which, on cleavage with dioxan–trichloroacetic acid, furnished 'petromyzonol', double m.p. about 225 and 240°, which was analysed by mass and n.m.r. spectroscopy. The resulting spectra strongly suggested the formula $C_{24}H_{42}O_4$ and the structure 5α-cholane-3α,7α,12α,-24-tetrol (Scheme 1, II) for petromyzonol, and this conclusion was fully confirmed when reduction with lithium aluminium hydride of the propyl ester (Scheme 1, III), of allocholic acid (3α,7α,12α-trihydroxy-5α-cholanoic acid, isolated from the bile



Scheme 1. Partial synthesis of petromyzonol and reactions of petromyzonol sulphate (see the text). Formulae (II) and (IV) show major mass-spectral cleavage patterns.

of two lizards of the family Iguanidae, gave a compound identical in all respects with petromyzonol.

The bile salts were oxidized with cold chromic acid and the product was hydrolysed to give a crystalline neutral substance, i.r.-, mass- and n.m.r.-spectral examination of which supported the structure 24-hydroxy-5α-cholane-3,7,12-trione (Scheme 1, IV).

All this evidence, taken together, puts beyond reasonable doubt the view that the principal or sole bile salt of *P. marinus* ammocoetes has the structure (I), i.e. is the C-24 sulphate ester of 5α-cholane-3α,7α,12α,24-tetrol.

EXPERIMENTAL

General. Methods and analyses were as described by Bridgwater, Briggs & Haslewood (1962). N.m.r. spectra were observed, with the solvents indicated, in a Varian HA100 spectrometer. Mass spectra were taken with an Atlas CH-4 mass spectrometer, equipped with a TO-4 ion source, at 70 ev ionization potential. T.l.c. was on films (0·25mm. thickness) of silica gel G (E. Merck A.-G., Darmstadt, Germany).

Isolation of petromyzonol

Preparation and properties of lamprey bile salts. An early sample of lamprey bile (in ethanol and possibly from *P. flaviatilis*, given by Dr P. D. G. Dean), gave a white solid (0·6mg.), which on paper chromatograms in the solvent system (S_1) pentyl acetate–heptane–acetic acid–water (17:3:21:10, by vol.) gave a single spot with an R_F greater than that of taurocholate and close to that of 5α-cyprinol sulphate. In later work, this appearance was taken to be indicative of lamprey bile salts, which were otherwise prepared from whole livers in which the gall-bladders were embedded. The principal source was a collection of about 200 such livers from *P. marinus*, preserved in a large excess of ethanol and supplied by Dr J. J. Tibbles. The livers were cut up and ground in a mortar with ethanol. Evaporation of the total filtered extract left a residue, which was triturated 4–5 times with light petroleum (b.p. 40–60°) to remove fats. The petroleum-insoluble residue was dissolved in cold methanol and the filtered solution evaporated to leave a solid (124mg.) giving a positive (purple) response to the Hammarsten (HCl) test and a single spot with mobility close to that of 5α-cyprinol sulphate on paper in system S_1 and on t.l.c. in the system pentyl acetate–acetic acid–water (5:5:2, by vol.). Two extractions of this solid (77mg.) with cold ethanol (about 1ml. for each extraction) left almost white crystalline material [42mg., m.p. 223° (decomp.)], containing traces of Cl^- and shown by flame photometry (by Mr B. Brambleby) to consist of approximately equal amounts (by wt.) of sodium and potassium salts of petromyzonol sulphate, whose i.r. spectrum in KBr differed somewhat from that of 5α-cyprinol sulphate but showed prominent absorption bands at about 9·2, 9·7, 9·9, 10·4 and 11·2μm.

Preparation of petromyzonol from lamprey bile salts. A solution of crude bile salts (47mg., prepared as described above) in acetic acid (0·5ml.) with acetic anhydride (0·5ml.)

was heated under reflux (with an air condenser) on a boiling-water bath for 1hr. Solvents were evaporated finally *in vacuo* at about 100°, and the residue was treated at once with 1ml. of a solution (40%, w/w) of dry trichloroacetic acid in dry dioxan. After about 20min. solution occurred and the product was left, with occasional mixing, for 28 days. It was then diluted with water and extracted three times with ether, after the addition of excess of NaCl. The aqueous portion, with 5ml. of 0·5M-$BaCl_2$, gave barium sulphate (16mg.). The ether extract was washed with water, aqueous ammonia and water, dried over Na_2SO_4 and evaporated. A solution of the residue in methanol (0·8ml.) was boiled under reflux for 1hr. with 5M-NaOH (0·2ml.) and solvent was then removed in a current of N_2. Water was added and the insoluble material, after refrigeration, was collected, washed with water and dissolved in methanol. Evaporation left a solid (18mg.) giving a single spot on paper chromatography in the system di-isopropyl ether–heptane–acetic acid–water (6:4:7:3, by vol.). This material was almost completely dissolved by heating with acetone. Evaporation of the filtered solution followed by treatment with a little fresh acetone gave colourless prisms (16mg.), which formed fine needles (10mg., m.p. 224–226°, partially recrystallizing and re-melting at 240–241°) from aqueous ethanol. This *petromyzonol* (Scheme 1, II) gave a positive (purple) Hammarsten (HCl) test and its i.r. spectrum (Fig. 1) showed the principal bands, mentioned above, characteristic of the allocholic acid nucleus.

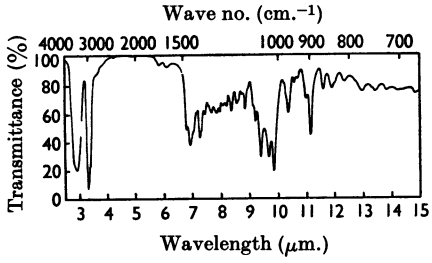


Fig. 1. Infrared spectrum, in KBr, of petromyzonol.

On paper chromatography in the system di-isopropyl ether–heptane–acetic acid–water (7:3:7:3, by vol.) it gave a single spot with the mobility of 5β-cholane-3α,7α,12α,24-tetrol. Results of its mass- and n.m.r.-spectral examination follow.

N.m.r. and mass spectra of petromyzonol. N.m.r. spectra of petromyzonol were measured in four different solvent systems. The positions of the angular-methyl-group resonances together with those of other bile salt derivatives are listed in Table 1. The values for petromyzonol are indicative of a 5α-configuration, with the possibility of 3α,7α,12α-trihydroxy substituents.

Analysis of the O*H* and C*H*·OH resonances was hindered by the overlap of these peaks and by the superimposed HDO resonance. However, integration of the peak areas in the spectrum in pentadeuteropyridine solution showed the presence of five protons in the C*H*·OH environment in the 3·5–4·5 p.p.m. region and about four OH protons in the 5·0–6·0 p.p.m. region. The chemical shifts and shapes (narrow multiplets) of three of the five C*H*·OH resonances indicated the presence of three equatorial protons, two of them (3β and 7β) resonating at 4·21 p.p.m. and one (12β) at 4·06 p.p.m. An unresolved two-proton multiplet centred around 3·8 p.p.m., which became a poorly resolved triplet when D_2O was added, is indicative of the presence of a $CH_2 \cdot CH_2 \cdot OH$ group.

In all spectra there was only one three-proton doublet (centred at 1·21 p.p.m. in pentadeuteropyridine, $J = 6$Hz) for a methyl group with one geminal hydrogen. This observation, together with the $CH_2 \cdot CH_2 \cdot OH$ resonance and the mass-spectral evidence given below, suggests that petromyzonol does not possess the cholestane side chain.

Table 1. *Proton resonances of the* C-18 *and* C-19 *methyl groups of some bile salt derivatives in different solvents*

Solvents	Resonances (p.p.m.)							
	Hexadeutero-dimethyl sulphoxide ([D_6]DMSO)		[D_6]DMSO–D_2O		Pentadeutero-pyridine		Heptadeutero-dimethylformamide	
Resonating protons	C-18	C-19	C-18	C-19	C-18	C-19	C-18	C-19
Compounds								
Methyl cholate (methyl 3α,7α,12α-trihydroxy-5β-cholanoate)	0·59*	0·81*	0·60*	0·82*	0·78	0·98	0·68*	0·88*
5β-Chimaerol (5β,25ξ-cholestane-3α,7α,12α,-24ξ,26-pentol)	0·59	0·82	0·60	0·82	0·81	0·98	—	
5α-Cyprinol (5α, cholestane-3α,7α,12α,-25,26-pentol)	0·58	0·67	0·60	0·685	0·795	0·885	0·67*	0·75*
Petromyzonol (structure II)	0·60	0·69	0·60	0·69	0·80	0·89	0·65	0·74

* These values are from 60MHz spectra; all other observations are from 100MHz spectra.

The mass spectrum of petromyzonol (II) exhibited a very small peak at *m/e* 376, a strong one at *m/e* 358 and a smaller one at *m/e* 340, due to the consecutive losses of three molecules of water. Considering that the n.m.r. and i.r. spectra showed no sign of unsaturation in the molecule, the most likely interpretation of these peaks is that they are due to the loss of one, two and three molecules of water respectively from an apparently unstable molecular ion of 394 mass units. This molecular ion is in agreement with an empirical formula of $C_{24}H_{42}O_4$. Peaks were also visible at *m/e* 343 and 325 due to the loss of a methyl radical from ions of mass 358 and 340 respectively.

Other peaks of diagnostic importance in the mass spectrum were at *m/e* 289, 271 and 253. These ions were formed by the loss of the side chain from the $M-H_2O$ ion (see formula II; a typical fragmentation of 12-hydroxy steroids) followed by the expulsion of two consecutive molecules of water. The sequence of the dehydration steps after the ring D cleavage were further substantiated by the observed metastable peaks at *m/e* 254 ($289^2/271=254{\cdot}1$) and 236 ($253^2/271=236{\cdot}2$). These results clearly establish the presence of three hydroxyl groups on the steroid skeleton (one of them being on C-12) and the presence of a $C_5H_{11}O$ substituent at C-17. This information, together with the n.m.r. results, is strongly suggestive of a structure as shown in Scheme 1 (II).

Petromyzonol from allocholic acid

Allocholic acid from Anolis lineatopus lineatopus *Gray and* Cyclura carinata *Harlan (family Iguanidae). Anolis lineatopus lineatopus* bile from 81 lizards, collected by Dr Garth Underwood in Jamaica, gave bile salts (153mg.) as a brown partly crystalline solid. This (143mg.) was heated in 2·5M-NaOH (1·4ml.) in a sealed metal bomb at 114° for 17hr. The clear brown solution was treated with 2M-HCl and excess of NaCl and, after refrigeration, the precipitated bile acids were collected. The yield, after evaporation of a filtered ethanolic solution, was 79mg. Two crystallizations from ethyl acetate gave material (22mg., m.p. 224–226°) chromatographically and spectroscopically indistinguishable from allocholic acid (Anderson & Haslewood, 1962). Evaporation of the ethyl acetate liquors left a residue (43mg.), which was dissolved in excess of methanol and methylated with excess of diazomethane. The resulting methyl esters on paper chromatography showed spots corresponding to methyl cholate (methyl 3α,7α,12α-trihydroxy-5β-cholanoate; not distinguished from methyl allocholate in the systems used) and methyl chenodeoxycholate (methyl 3α,7α-dihydroxy-5β-cholanoate), but none with the R_F of methyl trihydroxycoprostanate (methyl 3α,7α,12α-trihydroxy-5β-cholestan-26-oate). The methyl esters were separated on Celite (5g.) in the system light petroleum (b.p. 80–100°)–ethanol–water (7:5:2, by vol.). Moving phase (total, 220ml.) eluted three chief fractions as follows (fraction no., wt. in mg., ml. of effluent): I, 12·3, 0–20; II, 6·0, 22–42; III, 12·8, 122–220. Fraction I was a pigmented apparently non-steroid gum and fraction II on chromatography and i.r. spectroscopy was found to resemble methyl chenodeoxycholate: these fractions were not investigated. Fraction III and material (11·4mg.) stripped from the column with acetone (100ml.) were shown by Mr A. R. Tammar by t.l.c. in the system diethyl oxalate–dioxan (7:3, v/v) to consist of methyl allocholate with traces, in later runnings, of methyl cholate. Bile of *Cyclura carinata* (one specimen), also collected by Dr Underwood, gave bile salts (320mg.). These (254mg.) were similarly hydrolysed and worked up, by Dr I. G. Anderson, to yield bile acids (122mg.), which, with ethyl acetate, gave crystalline allocholic acid (74mg.).

Conversion of allocholic acid into petromyzonol. Allocholic acid (33mg., prepared from lizard bile as described above) was dissolved in propan-1-ol (0·5ml.) containing 2% (v/v) H_2SO_4. After standing overnight, the mixture was diluted with aqueous $NaHCO_3$ and extracted three times with ether. The ether extract was washed with water, dried over Na_2SO_4 and evaporated to yield (presumably) *n*-propyl allocholate (III, 30mg.). This material (28mg.) was dissolved in tetrahydrofuran (3ml., freshly distilled over $LiAlH_4$) and treated with $LiAlH_4$ (50mg., added gradually). The resulting gel was heated under reflux for 1hr., cooled and treated with ice and excess of 2M-HCl. The product was extracted thrice with a mixture of ether and ethyl acetate and the extract washed with water, dried over Na_2SO_4 and evaporated. The crystalline residue (25mg.) was heated under reflux with acetone and the filtered solution evaporated. With a little fresh acetone the residue formed 'sugary' crystals, m.p. 233–235°, which from aqueous ethanol gave small glistening prisms of *5α-cholane-3α,7α,12α,24-tetrol* (II), m.p. 229–231°, not depressed after admixture with petromyzonol, $[\alpha]_D+27{\cdot}5\pm2°$ (*c* 1·2 in ethanol) (Found: C, 72·2; H, 10·5; $C_{24}H_{42}O_4$ requires C, 73·1; H, 10·7%). The behaviour on chromatography and the i.r., n.m.r. and mass spectra of this material were identical with those of petromyzonol.

Conversion of petromyzonol sulphate into 24-hydroxy-5α-cholane-3,7,12-trione. Petromyzonol sulphate (29mg. of crystalline material described above) was dissolved by warming in acetic acid (0·3ml.) and the solution cooled to room temperature and treated with 20% CrO_3 (0·12ml., added gradually, with mixing, from a burette) until an orange colour persisted. After 30min., methanol (1ml.) was added and the mixture left for 1·5hr. After addition of a few ml. of freshly distilled aqueous formaldehyde, most of the acetic acid, methanol and formaldehyde was removed by steam-distillation, continued until the distillate was neutral to litmus. The residue was transferred to a dish and evaporated on a water bath to small bulk. A 1·5ml. portion of M-NaOH was added and the mixture filtered. The yellow filtrate was evaporated and a methanolic solution of the residue transferred to a small flask and evaporated. The residue in water (3ml.) with 2·5ml. of M-HCl was heated on a boiling-water bath under reflux for 19hr. An oil separated, becoming crystalline: after addition of excess of NaCl to the mixture and refrigeration, the solid was collected and washed with water. The aqueous liquors, with 1ml. of 0·05M-$BaCl_2$, gave barium sulphate (9·6mg.). The collected partly crystalline organic material was stirred with aqueous ammonia: it did not dissolve. It was re-collected, washed with water and dissolved in ethanol. Evaporation left a residue (8·4mg.), which was crystallized with ethyl acetate as fine white needles (4·3mg.) of *24-hydroxy-5α-cholane-3,7,12-trione* (IV), m.p. 194°, partially subliming and melting at 210–218°. The i.r. spectrum, in KBr, showed OH-stretching absorption. T.l.c. by Miss A. Dutton in the system trimethylpentane–ethyl acetate–acetic acid (5:5:1, by vol.) (Eneroth, 1963) showed a single strong spot with two faint additional spots.

The mass spectrum of this compound exhibited a molecular ion at *m/e* 388 and a dehydration fragment at *m/e* 370, indicating that three of the four hydroxyl groups of petromyzonol had been oxidized to carbonyl functions. The fragmentation pattern (formula IV, Scheme 1) was dominated by ring D cleavages. The loss of ring D with the transfer of a hydrogen atom to the charge-retaining fragment (*m/e* 261), the partial ring D cleavage (*m/e* 274) and the loss of the side chain (*m/e* 301) are typical fragmentation patterns of 12-oxo-steroids with a hydrogen atom at C-20 (Djerassi & Tökés, 1966). These cleavage products establish the location of the hydroxyl group on the side chain, and also that one of the three carbonyl functions is at C-12 and the other two are in rings A and B. There were no detectable ring A or B fission products in the spectrum, probably because the 3- and 7-oxo functions were not powerful enough to trigger fragmentation in competition with the 12-oxo group.

The C-18 and C-19 angular-methyl-group signals in the n.m.r. spectrum in deuterochloroform solution were at 1·07 and 1·37 p.p.m. respectively. These values are in good agreement with the calculated values (Bhacca & Williams, 1964) for both the 5α- (1·075 and 1·399 p.p.m.) and 5β- (1·075 and 1·407 p.p.m.) 3,7,12-trioxocholane derivatives; thus the choice of the 5α-configuration is based on the previous results with petromyzonol. Also visible were a three-proton doublet centred at 0·85 p.p.m. (J=6 Hz) and a two-proton multiplet around 3·6 p.p.m., which are due to the C-21 methyl group and C-24 protons respectively.

DISCUSSION

Chemical. Since no new stereochemistry is involved, partial synthesis of petromyzonol from allocholic acid and hence ultimately from cholic acid (Anderson & Haslewood, 1962) completely establishes its structure. The only other bile alcohol also fully known by partial synthesis is 5β-cyprinol (5β-cholestane-3α,7α,12α,26,27-pentol; Hoshita, Kouchi & Kazuno, 1963; Haslewood & Tammar, 1968), in which there is symmetry at C-25. In all other cases there is undetermined stereochemistry at C-24 or C-25 or both, or, as in the case of 5α-cyprinol, partial synthesis has not yet been achieved.

The fact that a neutral compound was the result of the reaction sequence (I)→(IV) provides satisfactory proof that C-24 is esterified in petromyzonol sulphate, and the structure of the oxidation product (IV) is established by its mass- and n.m.r.-spectral characteristics. These findings exclude the possibility of a second sulphate ester group in the lamprey bile salt molecule; such a group is present at C-3 in myxinol disulphate from hagfish bile.

The elucidation of the chemistry of petromyzonol is a good example of the power of n.m.r.- and mass-spectral methods, for without these consideration of the unexpected C_{24} formula might, on biogenetic grounds, have been much delayed.

Biological. As a bile salt, petromyzonol sulphate is both primitive and unique; primitive because it is an alcohol sulphate and unique because petromyzonol is the only known example of a C_{24} bile alcohol. All other bile alcohols, having 26 or 27 carbon atoms, can be considered as biogenetically nearer to cholesterol (C_{27}) than are the common C_{24} bile acids; the position of petromyzonol is more difficult to assess. In some animals (certain lizards, for example) making allocholic acid as their chief bile acid, the course of biosynthesis probably includes the stages: cholesterol → 5α-cholestane-3α,7α,12α-triol → 5α-cholestane-3α,7α,12α,26-tetrol → 3α,7α,12α-trihydroxy-5α-cholestan-26-al → 3α,7α,12α-trihydroxy-5α-cholestan-26-oic acid → 3α,7α,12α,24-tetrahydroxy-5α-cholestan-26-oic acid → 3α,7α,12α-trihydroxy-5α-cholanoic acid (allocholic acid) + propionyl-CoA (Danielsson & Tchen, 1968; Hoshita, Shefer & Mosbach, 1968). There is no point in this sequence at which 5α-cholane-3α,7α,12α,24-tetrol (petromyzonol) can arise; hence it must be formed either by reduction of the final C_{24} product, allocholic acid, or by some quite different biochemical process. Its formation from allocholic acid would imply the remarkable situation in which a biochemical pathway was followed in a primitive animal but had not been used again until a much later evolutionary period. On the whole it seems easier to believe that petromyzonol arises by a pathway unique to lampreys, involving an experiment in bile salt evolution not (as far as we know at present) carried out in other organisms. Both ideas of petromyzonol biogenesis ought, however, to be kept in mind, for the mechanisms of evolution at the molecular level are still poorly understood.

The other unusual feature of *P. marinus* bile salts is their simplicity; petromyzonol sulphate was the only compound detected. The physiological efficiency of this substance as an aid to digestion, absorption etc. is unknown, but it is evidently by itself adequate for the (perhaps not very formidable) problems presented by the diet of ammocoetes.

There is little chemical resemblance between hagfish and lamprey bile salts. The former have the 3β-hydroxyl group and complete carbon skeleton of cholesterol and have not, apparently, evolved to the 3,7,12-trihydroxy pattern basic to all other vertebrate types. Lamprey bile salts have this pattern and also the 3α-configuration; thus they seem biochemically more advanced. Such findings agree with generally accepted ideas about the two groups of cyclostomes; that they are not closely related and that the hagfishes represent the more primitive forms (Heintz, 1961).

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