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Comparative Studies of 'Bile Salts'

20. BILE SALTS OF THE COELACANTH, *LATIMERIA CHALUMNAE* SMITH*

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A preliminary examination of *Latimeria* bile salts showed that they were mainly of the alcohol sulphate type, that a mixture of at least three substances was present and that the principal bile alcohol gave an infrared-absorption spectrum indicating that it had a steroid nucleus different from that present in cholic acid or in the bile alcohols ranol and cyprinol (Haslewood, 1957). Because of the great rarity of the bile, obtained in the present instance from a coelacanth kept alive for some hours in captivity (Millot, 1955), we decided to postpone further work on it until techniques for the examination of bile alcohols had been improved and the chemistry of these substances was better understood.

We now report a detailed examination of *Latimeria* bile salts.

RESULTS

Cleavage of the dried bile salts with the dioxan–trichloroacetic reagent gave as the principal product an easily purified alcohol that we call 'latimerol'. Latimerol, m.p. 236°, $[\alpha]_D + 33°$, had an infrared-absorption spectrum (Fig. 1*a*) that was indeed different from those of substances containing either the cholic acid or the allocholic acid nucleus. However, the latimerol spectrum did show some of the bands (especially those at about 10·4 and 11·2 μ) that we have come to associate with the nucleus of allocholic acid (Anderson, Briggs & Haslewood, 1964), and, since latimerol gave a precipitate with digitonin solutions and also a purple colour in the Hammersten test, we thought that it might have the substituted ring structure of the unknown 3β,7α,12α-trihydroxyallocholanic acid. Attempts to make this acid from allocholic acid [3α,7α,12α-trihydroxyallo(5α)cholanic acid] and from other likely starting materials all failed, and we finally resorted to a method used by Danielsson, Kallner & Sjövall (1963) for the preparation of allodeoxycholic acids. This method [modified in turn from a procedure outlined by Chakravati, Chakravati & Mitra (1962)] involved in our case the partial epimerization (at C-5) of ethyl 7α,12α-dihydroxy-3-oxocholanate (I) by prolonged heating with Raney nickel in cumene. The resulting mixture

* Part 19: Haslewood (1964).

of esters was reduced with sodium borohydride in pyridine, a reagent reported (e.g. by Soloway, Deutsch & Gallagher, 1953) to give good yields of equatorial alcohols from the corresponding steroid ketones. After purification, as described below, an appropriate fraction of the ethyl esters resulting from this reduction was treated with a solution of digitonin, in the hope that only digitonides of 3β,5α-alcohols would be sufficiently insoluble to be precipitated. In fact, the precipitated digitonide was decomposed to give crude ethyl 3β,7α,12α-trihydroxyallocholanate (II), which, after purification on Celite, formed solvated crystals of m.p. 187° and $[\alpha]_D + 24°$, gave a purple colour in the Hammersten test and showed an infrared-absorption spectrum (Fig. 1*c*) containing all the principal bands between 9·0 and 15·0μ given by latimerol. Chromic oxidation of the acid derived from the ester (II) gave, in good yield, 3,7,12-trioxoallocholanic acid (III) (Anderson & Haslewood, 1962).

In addition to latimerol, the dioxan–trichloroacetic acid cleavage yielded a little cyprinol and a small amount of a partially purified substance whose behaviour on paper chromatograms was consistent with its being a hydroxyl derivative of latimerol. Inorganic sulphate was obtained, corresponding very roughly in amount to that calculated on the assumption that the bile alcohols isolated were originally present in the bile salts as monosulphates.

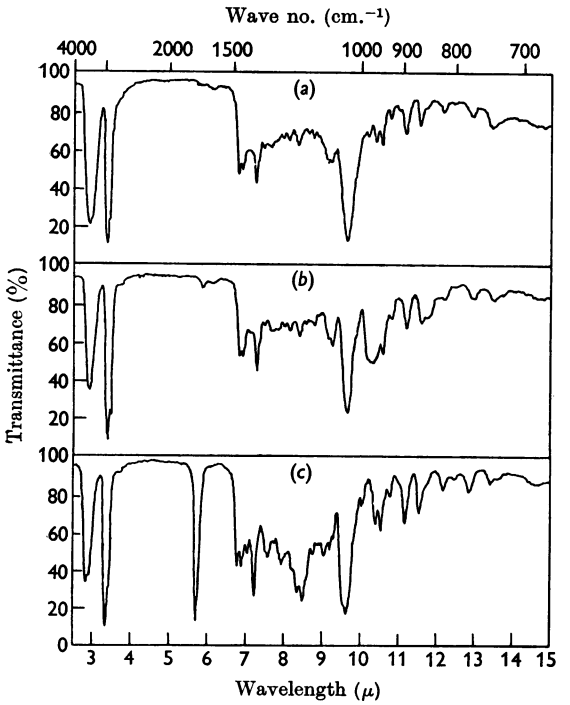


Fig. 1. Infrared-absorption spectra in potassium bromide of (*a*) latimerol, (*b*) anhydrolatimerol and (*c*) ethyl 3β,7α,-12α-trihydroxyallocholanate.

The preliminary examination of coelacanth bile salts (Haslewood, 1957) was done on material obtained after alkaline hydrolysis. When this material was re-examined, it was found to contain a little latimerol, but consisted chiefly of an alcohol, $C_{27}H_{46}O_4$, m.p. 185°, $[\alpha]_D + 31°$, whose infrared-absorption spectrum (Fig. 1*b*) was similar to that of latimerol, but showed greatly increased absorption at about 10·3μ. This feature, indicative of a trimethylene oxide ring, is characteristic of the 'anhydro' bile alcohols formed from monosulphates during alkaline hydrolysis by elimination of SO_4^{2-} ion between the $-O \cdot SO_3^-$ group and a suitably placed hydroxyl group (e.g. Anderson *et al.* 1964). The new alcohol $C_{27}H_{46}O_4$ is so related to latimerol and may be called 'anhydrolatimerol'. Mild chromic oxidation of anhydrolatimerol gave a substance identical with dehydroanhydrocyprinol (VI), similarly obtained from anhydrocyprinol (VII).

These results are interpreted to mean that latimerol has the formula (IV), that it occurs in *Latimeria* bile as the 27-monosulphate and that anhydrolatimerol, derived from this, has structure (V).

EXPERIMENTAL

General methods

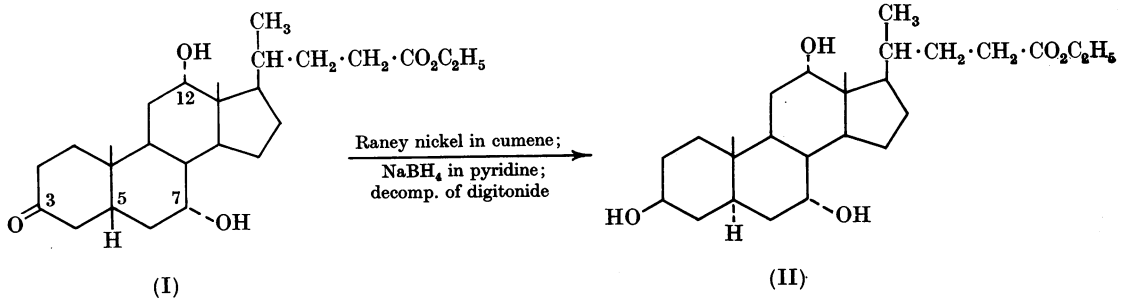
Methods for m.p. determination, chromatography on alumina, esterification, chromic oxidation, elementary analysis and infrared spectroscopy were as previously described (see Bridgwater, Haslewood & Watt, 1963).

Latimeria *bile salts*

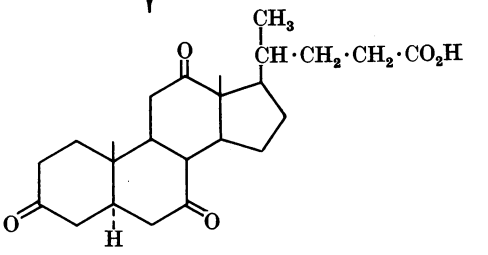
Bile, stated by Professor J. Millot to be one-half (15 ml.) of the contents of the gall-bladder of a coelacanth described by Millot (1955), was received as a clear solution in ethanol (excess). Evaporation left the bile salts (1·72 g.) as a hygroscopic brown powder.

Cleavage with dioxan–trichloroacetic acid

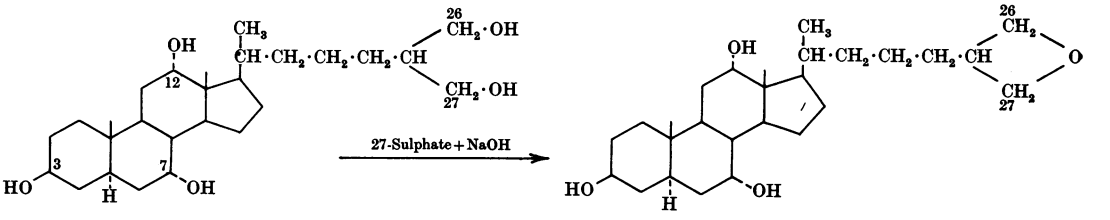
Crude *Latimeria* bile salts (0·3 g., dried *in vacuo* over $CaCl_2$) were dissolved by warming with acetic acid (3 ml.). Acetic anhydride (3 ml.) was added and the mixture heated under reflux on a boiling-water bath for 1 hr. The condenser was removed and solvents were evaporated in a stream of nitrogen. The residue was dried by evacuation at about 100°, stoppered and allowed to cool. The stopper was removed, and 3 ml. of a dry 40% (w/w) solution of freshly distilled trichloroacetic acid in dioxan was added immediately. The stoppered mixture was kept at room temperature with occasional shaking for 13 days; it was then diluted with water and extracted three times with ether. The ether was washed with water; the combined aqueous fraction and washings gave, after standing for some days with the addition of 0·5M-$BaCl_2$ (10 ml.), 76·3 mg. of



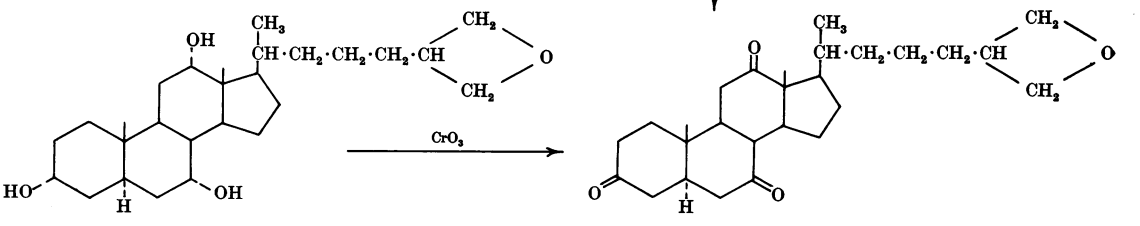
(I) (II)



(III)



(IV) Latimerol (V) Anhydrolatimerol



(VII) Anhydrocyprinol (VI)

$BaSO_4$ (equivalent to 181 mg. of a sulphate of formula $C_{27}H_{47}NaO_8S$). The ethereal extract was washed with aqueous ammonia and water, dried (over Na_2SO_4) and evaporated. The residue was a light-brown gum (192 mg.) which was dissolved in ethanol (5 ml.) and heated under reflux for 1 hr. with 5N-KOH (1 ml.). Ethanol was removed in a nitrogen stream and the residue diluted with water, collected and washed with water, giving an almost white solid (105 mg.) which was crystallized twice from acetone to give small glistening (solvated) prisms of *latimerol* (IV; 54 mg.), m.p. 235–236° (decomp.), $[\alpha]_D^{23}$ $+33\pm2°$ (*c* 1·1 in ethanol), infrared-absorption spectrum as in Fig. 1(*a*) (Found: C, 70·7; H, 10·4. $C_{27}H_{48}O_5$ requires C, 71·7; H, 10·6. $C_{27}H_{48}O_5,CH_3COCH_3$ requires C, 70·6; H, 10·6%.) This substance gave a purple colour in the Hammersten (HCl) test. Latimerol (about 2 mg.) was dissolved by warming with a portion (about 0·1 ml.) of a solution of digitonin [1% (w/v) in ethanol–water (2:1, v/v)]: on standing, this mixture deposited a gelatinous precipitate. On paper chromatograms in solvent system G_1 (Haslewood & Sjövall, 1954) latimerol gave a single spot running a little more slowly than cyprinol.

The residue left by evaporation of the acetone liquors from the first crystallization of latimerol showed on paper chromatograms in system G_1 spots corresponding to cyprinol and to latimerol, and a further spot with R_F about half that of latimerol. This mixture (20·4 mg.) was separated on Celite (10 g.) as described by Haslewood (1961) with the solvent system (EC_4): benzene–light petroleum (b.p. 80–100°)–ethanol–water (5:2:5:2, by vol.). Moving phase (benzene–light petroleum; 78–128 ml.) eluted a fraction (5·5 mg.) that had m.p. 234–237° (decomp.) not depressed by cyprinol and having the same infrared-absorption spectrum as that substance. A second fraction (8·1 mg.) eluted by moving phase (130–200 ml.) proved to be a mixture of cyprinol and latimerol. The Celite column was washed with methanol (100 ml.). Evaporation left a residue (4·3 mg., after washing with cold water) which on paper chromatograms in system G_1 gave a spot corresponding to latimerol and another with R_F about half that of latimerol and moving rather more slowly than scymnol. It would be expected that scymnol and allo(5α)scymnol would move at about equal rates and that the 3β,5α-epimer of scymnol would move more slowly than these compounds on paper chromatograms.

Alkaline hydrolysis of bile salts

In the original experiments (Haslewood, 1957), *Latimeria* bile salts (0·5 g.) in 2·5N-NaOH (10 ml.) were heated in a metal bomb at 118° for 5 hr. After cooling, the bomb was found to contain a gelatinous solid which was collected and washed with water. The filtrate and washings were acidified with HCl and extracted twice with ether (with 20 ml. of 0·5M-$BaCl_2$ the aqueous portion gave 77 mg. of $BaSO_4$). Evaporation of the washed and dried (over Na_2SO_4) ether extract left '*Latimeria* acids' as a brown gum (43 mg.), which were ethylated in the usual way: esterification was incomplete after 4 days. On paper chromatography in Bush's (1952) solvent system A, the esters (23 mg.) showed a spot with R_F about one-quarter that of ethyl cholate and a faint spot with the R_F of ethyl cholate.

The gelatinous alkali-insoluble precipitate was dried *in vacuo* over $CaCl_2$ to give '*Latimeria* neutrals' (131 mg.). Material similarly obtained in another experiment was continuously extracted in a Soxhlet apparatus with, in succession, (*a*) ethyl acetate, (*b*) acetone and (*c*) methanol, giving fractions (*a*) (62 mg.), (*b*) (19 mg.) and (*c*) (12 mg.). Fraction (*c*) apparently consisted chiefly of unhydrolysed bile salts and (*b*) contained a little impure latimerol, a further quantity (36 mg.) of which was also isolated after saturation of the original alkaline filtrate and washings with NaCl. Fraction (*a*) (43 mg.) was separated on Celite (10 g.) in solvent system EC_1 as described by Haslewood (1961). Moving phase (68–122 ml.) eluted a discrete fraction (23 mg.) giving a single spot with R_F about 0·5 on paper chromatography in solvent system G_3 (Haslewood & Sjövall, 1954). This material was crystallized with ether to give *anhydrolatimerol* (V), m.p. 184–185°, $[\alpha]_D^{19}+31\pm2°$ (*c* 0·8 in ethanol), infrared-absorption spectrum as in Fig. 1 (*b*). (Found: C, 74·2; H, 10·6. $C_{27}H_{46}O_4$ requires C, 74·6; H, 10·6%).

Dehydroanhydrocyprinol. Anhydrocyprinol (33 mg.; Anderson *et al.* 1964) in acetic acid (0·4 ml.) was treated gradually, with careful mixing, at room temperature with 20% CrO_3 (0·2 ml.). After 5 min., water (2 drops) was added to dissolve a precipitate and the solution left for a further 10 min. It was then treated with water and NaCl (excess) and extracted twice with ether. The ether was washed with water, aqueous ammonia and water, dried (over Na_2SO_4) and evaporated, leaving a crystalline residue (23 mg.) which was recrystallized from aqueous ethanol to give short white needles of *dehydroanhydrocyprinol* (VI), m.p. 193–195° (Found: C, 74·7; H, 9·4. $C_{27}H_{40}O_4$ requires C, 75·7; H, 9·4%: there was loss of weight on drying at 100°, but repeated analysis did not much raise the value for C content). Similar treatment of anhydrolatimerol (10 mg.), but with oxidation for a period of 1 hr., led to a substance of m.p. 190–195°, not depressed by dehydroanhydrocyprinol, and having the same infrared-absorption spectrum as that substance.

Preparation of ethyl 3β,7α,12α-trihydroxyallocholanate

Ethyl 7α,12α-dihydroxy-3-oxocholanate (I; 1 g.) was boiled for 16 hr. under reflux with cumene (isopropylbenzene; 88 ml., redistilled over KOH) and Raney nickel (made during 1·5 hr. from 12 g. of alloy, 16 g. of NaOH and 60 ml. of water at 50±2°, then washed with water until the washings were neutral to phenolphthalein, and finally successively with ethanol, dry benzene and cumene). Evaporation of the filtered solution left a gum (0·94 g.) which was dissolved in dry pyridine (7 ml.) and treated with powdered $NaBH_4$ (0·3 g.) at room temperature. After 16 hr. the mixture was treated with water and 2N-HCl (excess, added carefully). The product was extracted with ether, and the washed ether evaporated to leave a residue which was boiled under reflux in ethanol (14 ml.) with 5N-NaOH (1·4 ml.) for 50 min. Ethanol was evaporated and the residue mixed with water and extracted with ether, to remove a neutral oil. Ether was removed from the aqueous portion which was then treated with 2N-HCl and NaCl (excess) and cooled at 0–5°. The precipitated acid was collected, washed and ethylated in the usual way, giving solid ethyl esters (0·593 g.), which were dissolved in benzene and put on to a column of Al_2O_3 (5·9 g., neutralized) made up in benzene. Elution was as follows (fraction no., ml. of eluting solvent, mg. eluted): I, 125 ml. of benzene, 207; II, 100 ml. of ether, 48; III, 100 ml. of acetone, 293;

IV, 50 ml. of ethanol, 42. The total eluted was 590 mg. Fraction III (293 mg.) was dissolved by warming with 5 ml. of a digitonin solution [1% (w/v) in ethanol–water (2:1, v/v)], and the solution, which soon deposited a precipitate, was left at room temperature for 2–3 hr. and then at 0–5° overnight. The crystalline digitonide was collected, washed with ethanol–water (2:1, v/v), water and a little ether and dried to give a product (105 mg.) that was decomposed with pyridine and ether in the usual way to give an impure crystalline ester (25 mg.), m.p. 177–188°. The liquors from which the digitonide had been filtered, after evaporation and treatment with fresh digitonin, as above, gave a second crop (97 mg.) of digitonide, similarly yielding a crystalline ester (14 mg.), m.p. 182–184°. Combined crude crystalline ester (108 mg., prepared as above) was separated on Celite (10 g.) in solvent system EC_1 as described by Haslewood (1961). Moving phase (54–98 ml.) eluted a fraction (64·5 mg.) giving on paper chromatograms a single spot with R_F in solvent system B_3 of Bush (1952) about one-seventh that of ethyl cholate. With undried ether, this substance gave fine white (solvated) needles of *ethyl 3β,7α,12α-trihydroxyallocholanate* (II), m.p. 185–187°, $[\alpha]_D^{20}+24\pm2°$ (*c* 1·1 in ethanol). The anhydrous ester melted at about 148° and had an infrared-absorption spectrum as in Fig. 1 (*c*); it gave a purple colour in the Hammersten (HCl) test (Found: C, 71·9; H, 10·3. $C_{26}H_{44}O_5$ requires C, 71·6; H, 10·1%). This ester (approx. 20 mg.) was hydrolysed in the usual way to give a crystalline acid (10·6 mg.) that, without further purification, was dissolved in acetic acid (0·2 ml.) and oxidized with 20% CrO_3 (4 drops) for 30 min. at room temperature. Dilution with water and the addition of NaCl (excess) precipitated fine crystals that were collected, washed with water and recrystallized from aqueous ethanol to give long needles (8 mg.), m.p. 224–229° (decomp.) not depressed by authentic 3,7,12-trioxoallocholanic acid (III), and giving the same infrared-absorption spectrum as that substance.

The treatment described above of ethyl 7α,12α-dihydroxy-3-oxocholanate gave erratic results and frequently led to much smaller yields than that given above of the ester (II); reasons for this behaviour were not ascertained. Fraction I, eluted by benzene from Al_2O_3 as described above, also contained digitonin-precipitable material. Material (86 mg.) recovered from a digitonide isolated from this fraction was separated as described above on Celite (10 g.) in the solvent system (EC_3): light petroleum–ethanol–water (7:5:2, by vol.). Moving (light petroleum) phase (84–130 ml.) eluted a fraction (22 mg.) that with wet ether–light petroleum gave a *hydrate*, m.p. approx. 60–100°, recrystallizing and remelting at 144–145° (Found, on a sample dried to constant weight *in vacuo* at 100°: C, 74·5; H, 10·6. $C_{26}H_{42}O_4$ requires C, 74·6; H, 10·05%; the weight lost corresponded to 1 mol. of H_2O). This substance ran on paper chromatograms in Bush's (1952) system A as a single spot with R_F about 5 times that of ethyl cholate; it gave a faint (purple) response in the Hammersten (HCl) test and its infrared-absorption spectrum was similar to that of ethyl 3β,7α,12α-trihydroxyallocholanate (II) but lacked the band at about 11·2 μ (Fig. 1). These properties suggest that the new ester may possibly be an unsaturated dehydration product of (II), perhaps of the 'apocholic' type, i.e. an ethyl 3β,12α-dihydroxyallochol-*x*-enate.

DISCUSSION

Chemical. There seems to be little reasonable doubt that the epimerization product (II) really is ethyl 3β,7α,12α-trihydroxyallocholanate. The method of preparation, response to digitonin and to the Hammersten test, infrared-absorption spectrum and conversion into 3,7,12-trioxoallocholanic acid (III) are all in accordance with this view. The yield of (II) from (I) was poor and capricious; the method cannot be recommended as a general preparative route to allocholanic acids.

The chemical and infrared-absorption-spectral evidence that latimerol has the same nucleus as the ester (II) is entirely convincing.

Although latimerol cannot be converted into anhydrolatimerol, the isolation of these two substances as the major products of two different treatments of the bile salts strongly argues in favour of a direct chemical relationship between them. Elementary analysis, infrared spectroscopy and analogy with other bile alcohols provide good evidence that this relationship is expressed in formulae (IV) and (V). If this be accepted, the conversion of anhydrolatimerol (V) into dehydrocyprinol (VI) proves that latimerol has the carbon skeleton and hydroxyl-substitution pattern of cyprinol, a substance of known structure (Hoshita, Nagayoshi & Kazuno, 1963; Anderson *et al.* 1964).

In summary, the chemical evidence that latimerol is 3β,7α,12α,26,27-pentahydroxycholestane (IV) is very good; the conversion of its sulphate into anhydrolatimerol indicates that the native bile salt is the 27-sulphate ester of (IV).

Biological. Latimerol is the most 'primitive' (i.e. chemically nearest to cholesterol) bile alcohol so far discovered. Of course, it is possible that the 3β-hydroxyl configuration in this compound is a result of the action of intestinal micro-organisms during the enterohepatic circulation of the bile; however, this configuration is unknown in bile alcohols from other fishes and it seems unlikely (although not impossible) that its occurrence as an artifact should coincide with its presence in an animal of a very ancient type. It seems more probable that latimerol is indeed an early bile alcohol: a survivor from a time when a method of inversion of the hydroxyl group at C-3 in cholesterol had not been evolved in the ancestors of *Latimeria*. This view accords with the nature of *Latimeria* as a whole, and the presence of a little cyprinol in the bile suggests that some movement towards modernization has at least begun. It is proper to suspend judgement on the significance of the paper-chromatographic evidence for a small amount of bile acids, for in a carnivorous animal of this kind these could be derived from the diet.

The presence of a bile alcohol of the cyprinol type is in agreement with the view (e.g. Romer, 1945) that the coelacanths, although now marine, had a long history of freshwater life, for this kind of bile alcohol has so far been found only in fishes whose evolution is believed to have taken place in fresh water.

SUMMARY

1. Dioxan–trichloroacetic acid cleavage of the bile salts of the coelacanth, *Latimeria chalumnae* Smith, gave as principal product latimerol (3β,7α,12α,26,27-pentahydroxycholestane), a little cyprinol (3α,7α,12α,26,27-pentahydroxycholestane) and a very small proportion of a third (unidentified) bile alcohol. Alkaline hydrolysis of the bile salts gave a little latimerol, but chiefly anhydrolatimerol and a 'bile acid' fraction not characterized.

2. Anhydrolatimerol on mild chromic oxidation was converted into dehydroanhydrocyprinol, similarly obtained from anhydrocyprinol.

3. Allomerization (at C-5) of ethyl 7α,12α-dihydroxy-3-oxocholanate with Raney nickel in cumene, followed by reduction with sodium borohydride in pyridine, led to the isolation of the digitonin-precipitable ethyl 3β,7α,12α-trihydroxyallocholanate, which was converted by hydrolysis and chromic oxidation into 3,7,12-trioxoallocholanic acid. The infrared-absorption spectrum of ethyl 3β,7α,12α-trihydroxyallocholanate showed all the principal bands between 9·0 and 15·0 μ given by latimerol.

4. The bile salts of *Latimeria* are thus shown to be (*a*) the most primitive (i.e. the nearest chemically to cholesterol) of any so far examined, and (*b*) to be indicative (by virtue of their being of the cyprinol chemical type) of a freshwater history for the coelacanth.

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The Formation and Distribution of Methylamine in the Ruminant Digestive Tract

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Although it has long been known that ammonia is produced during ruminal fermentation (Mangold & Schmitt-Kramer, 1927; Lenkeit & Becker, 1938; Wegner, Booth, Bohstedt & Hart, 1941; Harris, Work & Henke, 1943), it was not until McDonald (1948) demonstrated its absorption from the rumen and subsequent return as urea in the saliva that the significant role of ammonia in rumen metabolism was fully appreciated. Since that time the formation, absorption and utilization of ammonia have received a great deal of attention, and it is now well established that ammonia is of major importance in the nitrogen metabolism of the ruminant animal.

In most of the previous work ammonia was estimated by the distillation of rumen liquor under alkaline conditions followed by acid–base titration,

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