Comparative Studies of 'Bile Salts'

12. APPLICATION TO A PROBLEM OF RODENT CLASSIFICATION: BILE SALTS OF THE CUTTING-GRASS, *THRYONOMYS SWINDERIANUS**

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The emphasis in these studies so far has been mainly chemical and analytical. With improved analytical methods and growing certainty about the chemical nature and distribution in animal species of at least the C_{24} bile acids, we feel that application of bile-salt comparisons to some biological problems may now be attempted. We report such an application in the following case.

Certain African rodents so strongly resemble animals in somewhat similar habitats in South America that some authorities have supposed them to be closely related. For instance, Simpson (1945) placed the West African cutting-grass, *Thryonomys swinderianus*, and the South American coypu, *Myocastor coypus*, in the same 'superfamily' (Octodontoidea). Wood (1955) discusses rodent classification and concludes that the placing of African forms like *Thryonomys* with the South American species, including the coypu, called by him 'Caviomorpha', is 'morphologically justified, but paleogeographically impossible', in view of the improbability of any geologically recent exchange of mammals between the continents.

The bile salts of the coypu have been carefully examined in this Laboratory (Haslewood, 1954), and we now report a similar investigation of those of the cutting-grass.

RESULTS

It was clearly shown that bile salts of the cutting-grass consisted largely of taurine conjugates. After hydrolysis, chenodeoxycholic (3α:7α-dihydroxycholanic) acid was identified, together with a smaller proportion of deoxycholic (3α:12α-dihydroxycholanic) acid. Ursodeoxycholic (3α:7β-dihydroxycholanic) acid was also detected. The very small ketonic fraction of the bile acids did not appear to contain 3α-hydroxy-7-oxocholanic acid. The bile salts could not be shown to contain any glycine conjugates.

EXPERIMENTAL

General. Melting points are corrected. The Al_2O_3 used was as previously described (Haslewood, 1958). Light petroleum was 'b.p. 40–60°', except where otherwise indicated.

Isolation of bile salts. Gall bladders from 10 specimens of cutting-grass gave by the usual method (Haslewood & Wootton, 1950) 0·76 g. of hygroscopic brown bile salts.

Examination of conjugates. With the system G_3 (for glycine conjugates) and by the methods of paper chromatography described by Haslewood & Sjövall (1954), no free bile acids or glycine conjugates could be detected, although as much as 520 μg. of bile salts was placed at one time on the start-line. With descending chromatography and the system T (for taurine conjugates) of Haslewood & Sjövall (1954), and also the 70% (v/v) acetic acid–water/80% (v/v) 'amyl acetate'–*n*-heptane system of Sjövall (1955), a single spot corresponding to taurodeoxycholate or taurochenodeoxycholate was detected.

Hydrolysis of bile salts and identification of bile acids. Bile salts (40 mg.) of the cutting-grass were heated in a sealed metal bomb with 2·5 N-NaOH (0·2 ml.) and water (0·2 ml.) at 112° for 6 hr. The product was diluted with water and the clear solution treated with 2 N-HCl and NaCl (excess). The semi-solid bile acids were collected, washed with water and dried by evaporation *in vacuo* with ethanol. The residue was left in 2·5% (v/v) H_2SO_4–ethanol (0·5 ml.) for 2·5 days. Water and $NaHCO_3$ (excess) were added and the mixture was extracted three times with ether. The ether was washed with water, dried (Na_2SO_4) and evaporated, leaving 'ethyl esters' (12 mg.).

Ethyl esters (286 mg.) were subjected to Girard separation as previously described (Haslewood, 1954). The 'ketones' (8·2 mg.) could not be identified. The 'non-ketones' (total wt. 272 mg.) on paper chromatography with Bush's (1952) system A gave a spot corresponding to ethyl deoxy- or chenodeoxy-cholate and a faint spot suggesting ethyl ursodeoxycholate or ethyl hyodeoxycholate (3α:6α-dihydroxycholanate).

'Ethyl esters' (12 mg.) in benzene (0·1 ml.) with light petroleum (0·2 ml.) were put on Al_2O_3 (0·5 g.) in a column. Elution was as follows [fraction letter, solvent (vol.), wt. eluted]: *A*, 2:1 (v/v) light petroleum–benzene (4 ml.), 6·4 mg.; *B*, benzene (4 ml.), 0·7 mg.; *C*, ether (10 ml.) 1·8 mg.; *D*, acetone (2 ml.), 0·2 mg.; *E*, ethanol (3 ml.), trace (total recovered, 9·1 mg.). Fractions were each examined by paper chromatography as previously described (Haslewood, 1954) with Bush's (1952) system A.

Only fraction *C* (ether eluate) gave a spot; this corresponded to ethyl deoxy- (or chenodeoxy-)cholate. Dr I. D. P. Wootton compared the infrared spectrum between 900 and 1300 cm.$^{-1}$ of material from fraction *C* with the spectra of mixtures of methyl chenodeoxy- and methyl deoxy-cholate (Wootton & Wiggins, 1953). The spectrum from fraction *C* corresponded to that from a mixture of

* Part 11: Haslewood (1958).

80% (w/w) chenodeoxycholate and 20% (w/w) deoxycholate.

In other experiments, ethyl ester 'non-ketones' were separated on Celite (Johns-Manville Co. Ltd., London, S.E. 11) columns, as described by Anderson, Haslewood & Wootton (1957). The moving phase was light petroleum (b.p. 84–95°) and the stationary phase was 90% (v/v) ethanol–water. The stationary phase (5 ml.) was put on the Celite (10 g.) by shaking it mechanically with the Celite and moving phase (70 ml.) in a stoppered flask for 1 hr. The columns, packed as previously described, were loaded with 'non-ketones' (40–45 mg.). Moving phase (50 ml.) now eluted a fraction (*P*) shown by paper chromatography to contain ethyl deoxy- or chenodeoxy-cholate. A further eluate of moving phase (250 ml.) gave a fraction (*Q*) considerably enriched in the more polar ethyl ursodeoxycholate. In this way, 'non-ketones' (171 mg.) were separated into fraction *P* (127 mg.) and fraction *Q* (44 mg.).

Fraction *P* (40 mg.) was dissolved in acetic acid (0·4 ml.) with acetic anhydride (0·1 ml.). One drop of 8·5 N-$HClO_4$ was added and the dark mixture was left with occasional shaking for 15 min. Water and NaCl (excess) were added and the product was extracted twice with ether. The ether was washed with aqueous NH_3 and water, dried (Na_2SO_4) and evaporated. The residue (43 mg.) crystallized from light petroleum in large colourless prisms (20 mg.), m.p. 103–105°, which after recrystallization from aqueous ethanol had m.p. 106–109°, not depressed by authentic ethyl diacetylchenodeoxycholate (m.p. 106–109°).

Fraction *Q* (44 mg.) was separated by paper chromatography as previously described (Haslewood, 1954) and gave material (4 mg.) which on paper chromatograms with Bush's (1952) system A showed a single spot corresponding to ethyl ursodeoxycholate; this ran distinctly faster than ethyl hyodeoxycholate. This material gave an infrared spectrum (examined through the kindness of Dr R. K. Callow) consistent with the view that it was impure ethyl ursodeoxycholate.

DISCUSSION

Coypu bile has been found to contain cholic (3α:7α:12α-trihydroxycholanic), chenodeoxycholic, ursodeoxycholic and 3α-hydroxy-7-oxocholanic acids, probably all conjugated mainly with glycine (Kazuno & Takuma, 1947; Haslewood, 1954; Haslewood & Sjövall, 1954). It is possible that all these bile acids except cholic and chenodeoxycholic acids are artifacts produced by microbial action in the intestinal tract (Norman & Sjövall, 1958), and this is also true of deoxycholic acid (Lindstedt & Sjövall, 1957) now found in cutting-grass bile. Perhaps, therefore, the only bile acids undoubtedly made in the liver from cholesterol in the two species under discussion are cholic acid and chenodeoxycholic acid; of these, chenodeoxycholic acid is common to both species, and cholic acid, which occurs in coypu bile in small amounts, might be detectable in cutting-grass bile if larger samples were examined.

Ignoring any possible significance (for classification) which the intestinal micro-organisms may have, we may consider the assessment that the 'native' bile salts of both rodent species discussed consist chiefly of chenodeoxycholic acid, with the difference that this is conjugated apparently entirely with taurine in the cutting-grass and largely with glycine in the coypu. This difference we believe to be biologically decisive.

Glycine-conjugated bile salts have been found only in mammals, and their distribution is 'patchy'. A very high proportion of such salts has been observed only in a few vegetarian species (for example, rabbit, coypu, pig). In large collections of coypu- and pig-bile salts from different sources, we have never found more than small proportions of taurine-conjugated compounds. In some other mammals we have not detected any glycine conjugates. Bremer (1956) demonstrated specificity in conjugating with glycine or taurine by liver microsomes from different species (rabbit, rat, chicken); the microsomes *in vitro* gave the same compounds as are known to occur in the bile salts.

It seems therefore unlikely that the kind of conjugation in either species now compared is much affected by diet; the conjugation actually found is almost certainly expressive of inherited liver-enzyme systems.

We think, therefore, that our results support the views of Wood (1955), and other authors quoted by him, that there is no close evolutionary relationship between the coypu and the cutting-grass. In further support of this view, it may be noted that the bile salts of the guinea pig (Imai, 1937), also placed in the Caviomorpha, closely resemble those of the coypu. It may be of interest to make further bile-salt comparisons of members of groups of rodents whose relationships are, morphologically, difficult to determine.

There is, of course, an inescapable element of speculation in any conclusion of this kind made from the nature of a chemical 'character'. A chemical character has the advantage of precision, but its history is unlikely to be revealed by evidence from fossil forms. Deductions made must be inferences from the state of affairs as they exist today; such deductions cannot exclude the possibility of mutations which might invalidate the arguments put forward above.

SUMMARY

1. The bile salts of the West African rodent *Thyronomys swinderianus* (the cutting-grass) have been found to consist chiefly of chenodeoxycholic acid conjugated with taurine. Smaller amounts of (conjugated) deoxycholic and of ursodeoxycholic acids were detected. No glycine conjugates could be found.

2. Comparison of the bile salts with those of the morphologically similar South American coypu (*Myocastor coypus*) previously examined (Haslewood, 1954) supports the view of Wood (1955) that the two rodent species are not closely related in an evolutionary sense.

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Studies of Sebum

9. FURTHER STUDIES OF THE COMPOSITION OF THE UNSAPONIFIABLE MATTER OF HUMAN-FOREARM 'SEBUM'*

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The unsaponifiable matter of human-forearm 'sebum' (skin-surface fat) has been shown to contain hydrocarbons (30–46 %), cholesterol (14–19 %) and wax alcohols (about 20 %). The hydrocarbon fraction contains 30–40 % of squalene and a normal chain paraffin. A sterol-like substance was detected in the unidentified fraction as were oxidation products of squalene (MacKenna, Wheatley & Wormall, 1950, 1952).

A further examination of the sterol fraction has now been made, and the composition of the wax alcohols has been studied by gas chromatography.

EXPERIMENTAL

Collection of 'sebum'. A bulk collection of sebum was obtained from normal male medical students by immersion of the forearms in acetone (twice-redistilled) as already described (MacKenna *et al.* 1952).

Isolation and fractionation of the unsaponifiable matter. The sebum was saponified as previously described (MacKenna *et al.* 1952) and the unsaponifiable matter isolated. The latter was then fractionated on grade II alumina (Brockman & Schodder, 1941) as described in previous work. In addition, however, the column was washed finally with acetic acid in an attempt to effect a more complete recovery.

Gas chromatography of the wax alcohols. A similar procedure was used to that described for the fatty acids of sebum (James & Wheatley, 1956). The column used had as stationary phase Apiezon M vacuum grease (Shell Chemicals Ltd.) on Celite 545 (Johns-Manville Co. Ltd.) (2·5 parts of Apiezon M to 10 parts of Celite, 40–90 mesh) and was maintained at a temperature of 197° with boiling ethylene glycol. The sample (4·6 mg.) was applied to the top of the column with a micropipette and a flow rate of 60 ml. of N_2/min. was used. Tentative identification of the peaks followed the procedure used for the identification of the fatty acids of sebum (James & Wheatley, 1956); the relative retention times of peak emergence used in these identifications were calculated with reference to octadecanol (cf. James & Martin, 1956). Unsaturated alcohols were removed by the bromination method (James & Martin, 1956) and a second run was made of the saturated members only. Peak areas were measured by the triangulation method and corrected to octadecanol as described by James & Wheatley (1956). From the corrected areas the proportions of each component were calculated.

* Part 8: Boughton, MacKenna, Wheatley & Wormall (1957).

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