Farmer, T. H. (1959). *Biochem. J.* **73**, 321.
Farmer, T. H. & Morris, C. J. O. R. (1956). *Nature, Lond.*, **178**, 1465.
Gross, D. (1956). *Nature, Lond.*, **178**, 29.
Hayes, E. E. & White, W. F. (1954). *Recent Progr. Hormone Res.* **10**, 265.
Heilmann, J., Barollier, J. & Watzke, E. (1957). *Hoppe-Seyl. Z.* **309**, 219.
Hill, R. L. & Smith, E. L. (1957). *J. biol. Chem.* **228**, 577.
Morris, C. J. O. R. (1951). *Analyst*, **76**, 470.
Sayers, M. A., Sayers, G. & Woodbury, L. A. (1948). *Endocrinology*, **42**, 378.
Shepherd, R. G., Willson, S. D., Howard, K. S., Bell, P. H., Davies, D. S., Davis, S. B., Eigner, E. A. & Shakespeare, N. E. (1956). *J. Amer. chem. Soc.* **78**, 5067.

Biochem. J. (1961) **78**, 352

Comparative Studies of 'Bile Salts'

13. BILE ACIDS OF THE LEOPARD SEAL, *HYDRURGA LEPTONYX*, AND OF TWO SNAKES OF THE GENUS *BITIS**

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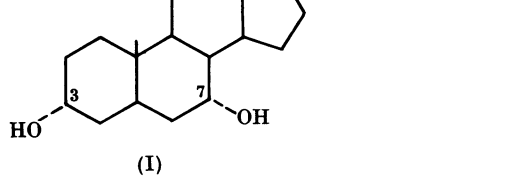
In the preliminary survey with which this work began the bile of ten species of snakes was examined. The Boidae (three species) gave pythocholic lactone (3α:12α:16α-trihydroxycholanic acid lactone), six other species yielded cholic acid (3α:7α:12α-trihydroxycholanic acid), but from the Gaboon viper, *Bitis gabonica*, no known bile acid was isolated (Haslewood & Wootton, 1950). An investigation of the bile salts of this species by paper chromatography (Haslewood & Sjövall, 1954) confirmed their unusual nature.

The work now reported included a detailed chemical examination of the bile salts of the Gaboon viper and also those of the puff adder, *Bitis arietans*, a closely related species. It was found that the bile salts of these snakes resembled those of Pinnepedia. For comparison, therefore, the leopard seal, *Hydrurga leptonyx*, was also investigated. Some knowledge was, incidentally, obtained of the bile acids of the Californian sealion, *Zalophus californianus*.

RESULTS

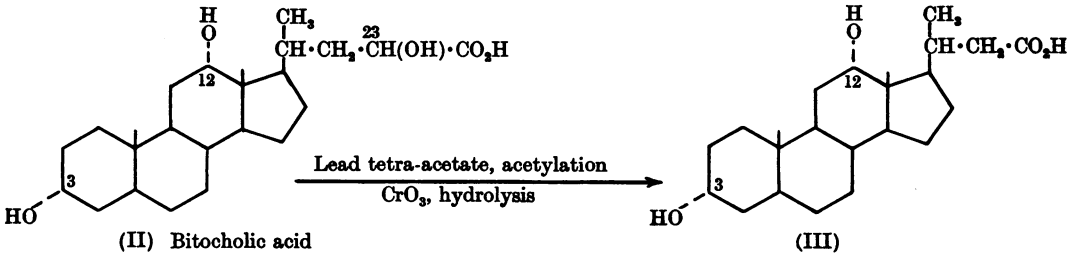
Paper chromatograms of the methyl or ethyl esters of Gaboon-viper bile acids showed faint spots corresponding to those given by methyl or ethyl cholate, together with two additional intense spots. In the solvent systems used, one of these intense spots (P) appeared just behind the methyl or ethyl cholate spot, and the other (Q) moved only a little way from the start-line. Fortuitously it was noticed that spots P and Q appeared at the same places on chromatograms as spots given by the corresponding esters of leopard-seal and Californian-sealion bile acids. Chromatography on alumina of the ethyl esters of Gaboon-viper bile acids easily separated the fraction responsible for spot P from a 'spot Q–ethyl cholate' fraction, described later.

The ethyl ester giving spot P was hydrolysed and the (non-crystalline) acid was investigated, with the clue that it might be related to the bile acids of Pinnepedia. The characteristic chemical feature of these acids is the C-23 hydroxyl group, found by Windaus & van Schoor (1928) in 'β-phocaecholic acid' (3α:7α:23-trihydroxycholanic acid, I). This acid was now isolated from leopard-seal and Californian-sealion bile and conditions were found by which the –CH(OH)·CO_2H grouping could be quantitatively estimated by lead tetra-acetate oxidation. Application of this method to the 'spot P' of Gaboon-viper acid gave the same result, quantitatively, as had been obtained from 'β-phocaecholic acid' (I). However, the 'spot P' acid did not crystallize with (I), and its specific rotation was $[\alpha]_D + 48{\cdot}5°$, in contrast with $[\alpha]_D + 11°$ for 'β-phocaecholic acid'. These facts, and the



(I)

* Part 12: Haslewood & Ogan 959).



(II) Bitocholic acid (III)

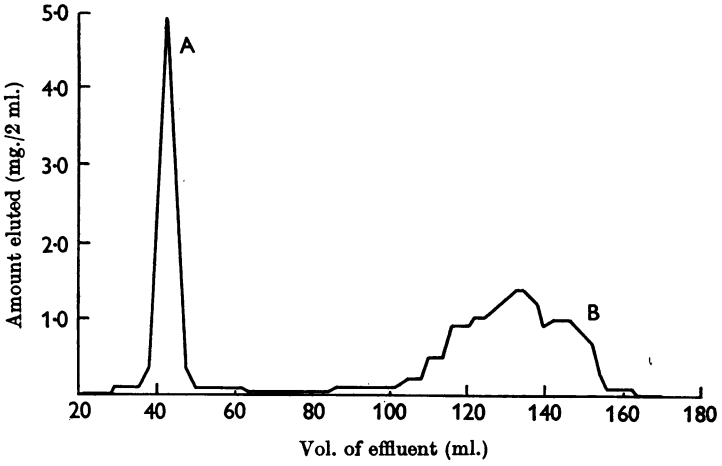


Fig. 1. Results of separating the complex (40 mg., m.p. about 205°) of ethyl esters from Gaboon-viper bile by partition chromatography on Celite (10 g.). Peak A is due to the ethyl ester of an isomer of cholic acid and peak B to ethyl 3α:7α:12α:23-tetrahydroxycholanate. Details are as given in the text.

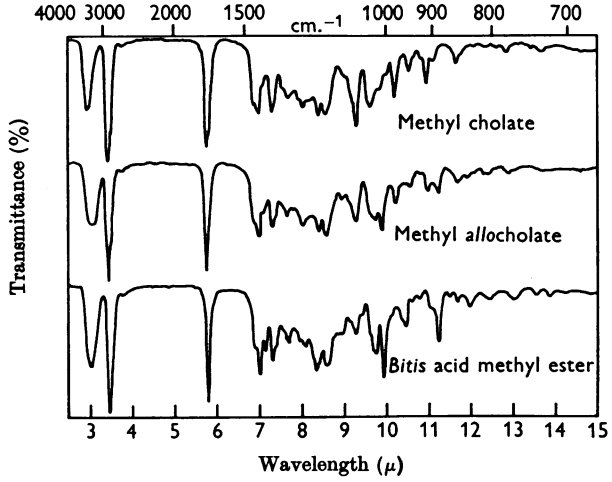


Fig. 2. Infrared spectra in KBr of methyl cholate, methyl *allo*(5α)cholate and the methyl ester of the cholic acid isomer, from Gaboon-viper and puff-adder bile, whose ethyl ester gave peak A (Fig. 1). Spectra were recorded separately and are redrawn together for comparison. In each case the percentage transmittance at 2·5 μ was above 94.

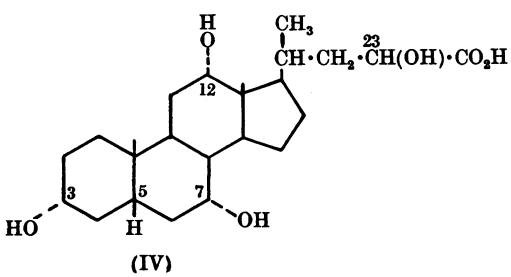
finding that the methyl or ethyl esters of the 'spot P' acid ran on chromatograms at the same rate as the corresponding esters of (I), suggested that the new acid might be 3α:12α:23-trihydroxycholanic acid (II), which would be expected to have the properties described. A larger sample of the acid giving spot P was therefore oxidized with lead tetra-acetate and the product was acetylated and further oxidized with chromic acid. From the saponified product there was obtained nordeoxycholic acid (III), identical with a sample made from deoxycholic acid (3α:12α-dihydroxycholanic acid) by the method of Riegel, Moffett & McIntosh (1944). These experiments show that a chief constituent of Gaboon-viper bile acids is (II), which it is proposed to call *bitocholic acid*. Methyl bitocholate was readily obtained crystalline as its complex with diethyl ether.

For confirmation of the formula (I) of Windaus & van Schoor (1928), 'β-phocaecholic acid' was degraded by the above-described methods (as II → III) to give norchenodeoxycholic acid, also prepared by Wieland–Barbier degradation of chenodeoxycholic acid (3α:7α-dihydroxycholanic acid).

The 'spot Q–ethyl cholate' fraction mentioned above was crystallized from ethyl acetate to give a substance melting constantly at about 205° and giving an elementary analysis corresponding to a formula $C_{26}H_{44}O_5$ or $C_{26}H_{44}O_6$. Paper chromatography of this substance showed it to be a complex containing an ester with the mobility of ethyl cholate and another giving spot Q. Separation on Celite gave the result shown in Fig. 1.

Elution peak A (Fig. 1) was due to an isomer (m.p. 225°, $[\alpha]_D + 23°$) of ethyl cholate which gave a positive Hammarsten (HCl) test. The corresponding methyl ester had m.p. about 227° and the acid melted at about 240°. The infrared spectra of the new methyl ester, of methyl *allo*(5α)cholate and of methyl cholate are shown in Fig. 2.

Peak B (Fig. 1) was given by an ester which crystallized as a hydrate, m.p. 182°, $[\alpha]_D + 36°$, and on saponification gave an acid with the properties expected of 3α:7α:12α:23-tetrahydroxycholanic acid (IV). Bergström, Krabisch & Lindeberg (1959) claim that the 'α-phocaecholic acid' of Hammarsten (1909, 1910) has this constitution. The infrared spectrum of the 'α-acid' was almost but not quite identical with that of the tetrahydroxy acid from the Gaboon viper. Probably both the acid of Bergström *et al.* (1959) and the Gaboon viper tetrahydroxy acid consist mainly of 3α:7α:12α:23-tetrahydroxycholanic acid (IV), and elution peak B (Fig. 1) is due to ethyl 3α:7α:12α:23-tetrahydroxycholanate. Some evidence was obtained that Gaboon-viper bile contained a little cholic acid.



(IV)

An identical complex, m.p. about 205°, was isolated from the ethyl esters of puff-adder bile acids and was resolved in the same way and with the same results as shown in Fig. 1. The bile of the puff adder also yielded methyl bitocholate.

Leopard-seal and Californian-sealion bile yielded 'β-phocaecholic acid', and from the leopard-seal ethyl esters there was obtained a complex, m.p. about 205°, having almost the same elementary analysis and infrared spectrum as the corresponding substance from the Gaboon viper. Resolution of this complex on Celite gave a result very similar to that shown in Fig. 1, and the ester corresponding to elution peak B (Fig. 1) was also ethyl 3α:7α:12α:23-tetrahydroxycholanate. The ester eluted at the position of peak A (Fig. 1) was, however, ethyl *allo*(5α)cholate [ethyl 3α:7α:12α-trihydroxy(5α)-cholanate], previously prepared from cholic acid (Anderson & Haslewood, 1960), and there was no evidence for the presence of the ester, m.p. 225°, described above.

Attempts to detect the value and sign of the contribution to $[\alpha]_D$ of the ·CH(OH)· group at C-23 in 'β-phocaecholic acid' by comparing the specific rotations of this acid, its ethyl ester and its acetylated ethyl ester with the rotations of the corresponding derivatives of chenodeoxycholic acid failed, since, within the limits of experimental error, the contribution to $[\alpha]_D$ of the asymmetry at C-23 could not be detected.

EXPERIMENTAL

General. Details were as described by Haslewood & Wootton (1951), except that melting-point determinations and esterification of bile acids were done as by Haslewood (1956). Paper chromatography was as described by Haslewood (1956), and partition chromatography on Celite as by Haslewood & Ogan (1959). Infrared spectroscopy in the range 2·5–15·0 μ was done with a Perkin–Elmer Infracord apparatus, by the KBr-disk method. A solution of the steroid (0·5–1·0 mg.) in acetone (about 0·2 ml.) was dropped on to powdered KBr (320 mg., Merck's 'for infrared spectroscopy') in a mortar and the solvent was evaporated at 50°. The KBr was re-ground and a disk prepared in the usual way. The use of acetone ensured the removal of the solvent frequently present in crystalline bile acids and their derivatives.

Lead tetra-acetate oxidation (quantitative method). The substance to be tested (1·0–4·0 mg.) was dissolved at about 50° in 1 ml. of 0·01 M-lead tetra-acetate in acetic acid, a blank (without substance) being prepared at the same time. After 16–18 hr. at 54°, there was added to each mixture 1 ml. of an aqueous solution containing sodium acetate trihydrate (50 g.) and KI (2 g.)/100 ml. (Baer, 1940). The liberated iodine was titrated with 0·01 N-$Na_2S_2O_3$. With this method, phocaecholic acid ('β-phocaecholic acid') consumed, compared with the blank, 1 equiv. of oxygen (error in three successive determinations, 0–+0·3%).

Preparation of esters. Bile salts (1 g.) were hydrolysed by heating in a small bomb at 114±2° for 8–16 hr. with 2·5 N-KOH (10 ml.). The contents of the bomb were washed out with water and treated with 2 N-HCl and NaCl (excess). After some hours at 0–5°, the solid crude bile acids were collected, washed with water and esterified as described above.

Separation of the ethyl esters of Gaboon-viper and puff-adder bile acids

The description below applies to both species, except where otherwise stated.

Preliminary paper chromatography. The ethyl esters in system B_3 of Bush (1952) gave a faint spot at the same place as ethyl cholate, a strong spot (P), just behind this and another (Q) just moving from the start-line. Neither P nor Q appeared at the same place as spots given by pythocholic lactone or ethyl varanate (Haslewood & Wootton, 1950) run on the same chromatogram. Similar results were given by the methyl esters of Gaboon-viper bile acids, except that spot P appeared at the same place as the spot due to pythocholic lactone. P and Q, whether due to ethyl or methyl esters, appeared at the same places as spots derived from the corresponding esters of leopard-seal or Californian-sealion bile acids.

Separation of ethyl esters on alumina. The esters (0·5 g.) in benzene (about 30 ml.) were put on to a column of Al_2O_3 (5 g.) made up in benzene. Elution was as follows (eluting solvent, ml., mg. eluted): benzene, 1000, 215; ether, 250, 12; ethanol, 250, 234. Total eluted, 461 mg. Paper chromatography showed that the benzene-eluted fraction gave only spot P, and that this was missing from the ethanol ('ethyl cholate–spot Q') fraction. In subsequent work, only the 'spot P' and 'ethyl cholate–spot Q' fractions were used, the small fractions eluted by ether being discarded.

Bitocholic acid. A fraction (194 mg.) giving only spot P, eluted by benzene as described above, was boiled under reflux for 40 min. in ethanol (3 ml.) with 5 N-KOH (0·5 ml.). Ethanol was removed by evaporation in N_2 and a solution of the residue in water was filtered. The filtrate was treated with 2 N-HCl and NaCl (excess). After cooling to 0–5° overnight, the solid acid was collected, washed with water and dried by evaporation with ethanol *in vacuo*. Yield, 171 mg. This bitocholic acid formed a colourless 'solid foam' which gave the same result as phocaecholic acid in the lead tetra-acetate method, but would not crystallize on seeding in ethyl acetate with this acid. Treatment of crude bitocholic acid in methanol with diazomethane (excess), followed by evaporation of solvent, left a gum which readily crystallized from ether as large colourless prisms of the ether complex of *methyl bitocholate*, m.p. 114–123°; $[\alpha]_D^{23}$ +44·2±1° in ethanol (*c*, 0·34) (Found: C, 70·4; H, 10·2. $C_{25}H_{42}O_5,C_4H_{10}O$ requires C, 70·2; H, 10·5%). This substance would crystallize only as the complex from ether: it gave a single spot as 'spot P' on paper chromatograms. Saponification of crystalline methyl bitocholate complex gave bitocholic acid, finally as a colourless gum with $[\alpha]_D^{23}$ +48·5±1° in ethanol (*c*, 1·3), which gave no colour in the Hammarsten HCl test.

Isolation and resolution of the complex m.p. about 205°. Combined fractions (497 mg.) of ethyl esters eluted by ethanol from Al_2O_3 in the separation described above were crystallized twice from ethyl acetate to give fluffy white needles of m.p. 202–206° (Found: C, 70·0; H, 9·7. $C_{26}H_{44}O_5$ requires C, 71·6; H, 10·1; $C_{26}H_{44}O_6$ requires C, 69·0; H, 9·7%). On paper chromatography with the solvent system G_3 of Haslewood & Sjövall (1954) this substance gave a spot at the same place as ethyl cholate (R_F 0·79) and another (Q) with R_F 0·24. The mixture (40 mg.) was separated on Celite with the system (EC_1) ethanol–water–light petroleum (b.p. 80–100°)–benzene (10:4:7:7, by vol.). Celite (Johns-Manville Co. Ltd., London) (10 g.) was impregnated with stationary (ethanol–water) phase (5 ml.), and, after packing, the above-mentioned complex in stationary phase (0·5 ml.) was put on to the column as described by Haslewood & Ogan (1959). The eluate with the moving phase was automatically collected in fractions (2 ml. each) into weighed tubes, with the result shown in Fig. 1.

Isolation of substance giving peak A (Fig. 1). Fractions eluted by between 40 and 46 ml. (both inclusive) were combined and the total crystalline residue (13 mg.) was recrystallized from aqueous ethanol–acetone to give white leaflets (10·5 mg.) of m.p 224–226°; $[\alpha]_D^{21}$ +23±2° in ethanol (*c*, 1·1) (Found: C, 71·75; H, 10·3. $C_{26}H_{44}O_5$ requires C, 71·6; H, 10·1%). This *substance*, like ethyl cholate, gave a purple colour in the Hammarsten HCl test. It (3 mg.) on saponification gave the corresponding acid, which slowly formed, from ethyl acetate, long needles of m.p. 239–241° (decomp.). The methyl ester, made with diazomethane, crystallized from ether as needles of m.p 224–227°: its infrared spectrum is shown in Fig. 2. It ran on paper chromatograms, in system G_3, at the same rate as methyl cholate and methyl *allo*cholate. There was insufficient pure material for further elementary analyses.

Isolation of substance giving peak B (Fig. 1). Fractions eluted by between 110 and 152 ml. (inclusive) were combined. The product (27 mg.) from (undried) ethyl acetate formed a gel which slowly became fine colourless needles of m.p. 184–185°; $[\alpha]_D^{27}$ +36±1° in ethanol (*c*, 1·1) (Found: C, 67·3; H, 9·4. $C_{26}H_{44}O_6,\frac{1}{2}H_2O$ requires C, 67·7; H, 9·8%). This (probably) *ethyl* 3α:7α:12α:23-*tetrahydroxycholanate* gave a greenish purple in the Hammarsten–HCl test. Saponification gave an amorphous acid, which consumed 1 equiv. of oxygen in the lead tetra-acetate method and gave an infrared spectrum almost identical with that of a specimen of Hammarsten's 'α-phocaecholic' acid supplied by Professor S. Bergström.

Evidence for cholic acid in Gaboon-viper bile. The ethyl acetate liquors from which the complex, m.p. about 205°, had been crystallized were evaporated, leaving a gelatinous solid. When this (50 mg.) was separated on Celite as described above there was obtained, in the place corresponding to peak A (Fig. 1), a substance (3·5 mg.) which had m.p. about 160° and appeared to be an impure sample of ethyl cholate. The substance (44·4 mg.) recovered in the peak B region (Fig. 1) was apparently ethyl 3α:7α:12α:23-tetrahydroxycholanate.

It could be calculated that constituents of the Gaboon-viper bile acid esters were present approximately in the following proportions (by wt.): ethyl bitocholate, 43%; ethyl 3α:7α:12α:23-tetrahydroxycholanate, 44%; ester m.p. 225°, 3%; ethyl cholate, 3%.

Bile acids of the leopard seal and Californian sealion

Leopard-seal bile acids and esters. The bile salts (8·2 g. from one gall-bladder) consisted of a non-deliquescent buff solid. When this (1 g.) was hydrolysed as described above, the crude bile acids (0·75 g.) formed an almost white solid. Esterification of this (3·77 g.) as previously described gave ethyl esters (3·16 g.), which, after treatment with Girard T reagent (Haslewood, 1956), yielded 'ketones' (0·02 g.) and 'non-ketones' (2·92 g.). The gummy 'ketones' were not further investigated.

Separation of ethyl esters on alumina. The esters (1·89 g.) in benzene (about 20 ml.) were put on to Al_2O_3 (19 g.) in a column. Benzene (1 l.) eluted 1·10 g., ether (250 ml.) eluted 0·05 g. and ethanol (250 ml.) eluted 0·64 g. Total eluted, 1·79 g. Paper chromatography, in Bush's (1952) system *A*, showed that the fractions eluted by benzene and ether both gave a strong spot (P) corresponding to ethyl phocaecholate and a faint spot running slightly more slowly than ethyl chenodeoxycholate. In Bush's (1952) system B_3, the fraction eluted by ethanol gave a strong spot (Q) running much more slowly than ethyl cholate and a faint spot at the same place as ethyl cholate.

Isolation and properties of phocaecholic acid. Combined benzene and ether eluates (1·70 g.) were heated for 20 min. under reflux in ethanol (50 ml.) with 5N-KOH (3 ml.). Ethanol was evaporated *in vacuo* and the residue was dissolved in water and treated with 2N-HCl and NaCl (excess). After cooling to 0–5°, the solid acids were collected, washed and crystallized twice from ethyl acetate, to give phocaecholic acid (0·5 g.), final m.p. 222–224°; $[\alpha]_D^{24}+10·8\pm1°$ in ethanol (*c*, 2·8). Ethyl phocaecholate (not crystalline) had $[\alpha]_D^{24}+10·4\pm1°$ in ethanol (*c*, 2·7). This ester (32 mg.) was left in pyridine (0·5 ml.) with acetic anhydride (0·5 ml.) for 2 days. The mixture was diluted with 2N-HCl and the product was extracted twice with ether. The extract was washed with water, aqueous ammonia and water and dried (Na_2SO_4). Evaporation left a crystalline residue of *ethyl triacetyl phocaecholate*, which gave large crystals, m.p. 165–171°, from light petroleum, and from aqueous ethanol gave small white hydrated needles, becoming amorphous at 144°, then crystallizing and finally melting at about 170°; $[\alpha]_D^{22}+18·7\pm1°$ in ethanol (*c*, 2·0) (Found, after drying: C, 68·2; H, 9·2. $C_{32}H_{50}O_8$ requires C, 68·3; H, 8·9%). Crystalline methyl chenodeoxycholate had $[\alpha]_D^{20}+11·2\pm1°$ in ethanol (*c*, 2·8) and the diacetate of this ester had $[\alpha]_D^{22}+17·5\pm1°$ in ethanol (*c*, 2·1). Corresponding fractions from Al_2O_3 prepared in this laboratory by Dr R. J. Bridgwater of the ethyl esters of Californian-sealion bile acids similarly yielded phocaecholic acid.

Complex m.p. about 205° from the leopard seal. The fraction of ethyl esters eluted from Al_2O_3 by ethanol (above) was crystallized twice from ethyl acetate, from which it gave long white needles, m.p. 201–204°, not depressed by the corresponding substance from Gaboon-viper bile (Found: C, 69·6; H, 10·1%). The compound had almost the same infrared spectrum as the Gaboon-viper substance and 13 mg. was separated on Celite exactly as described above. The peak A (Fig. 1) ester (5 mg.) appeared from aqueous ethanol as a gel, turning gradually into fine white needles, m.p. 204–205°; Hammarsten test, blue. Its infrared spectrum was identical with that of ethyl *allo*cholate, similarly separated from a complex with ethyl 3α:7α:12α:23-tetrahydroxycholanate (Anderson & Haslewood, 1960). The peak B (Fig. 1) ester (8 mg.) from the leopard-seal complex formed from ethyl acetate a gel which became long white needles of m.p. 181–183°, not depressed by ethyl 3α:7α:12α:23-tetrahydroxycholanate isolated as described above from snake bile.

Conversion of bitocholic acid into nordeoxycholic acid

Bitocholic acid (38 mg.) was dissolved in 3 ml. of 0·05M-lead tetra-acetate in acetic acid. The solution was kept at 50° for 18 hr., after which time iodine was liberated with the sodium acetate–KI reagent (3 ml.) described above. Titration showed that more than 1 equiv. of oxygen had been consumed. Excess of 0·05N-$Na_2S_2O_3$ and NaCl were added. The product was extracted twice with ether and the extract washed with water and dried (Na_2SO_4). Evaporation left an almost colourless gum (40 mg.), which was dissolved in acetic acid (1 ml.) with acetic anhydride (0·2 ml.). The solution, cooled to about 20°, was treated with 8·5N-perchloric acid (1 drop). After 10 min., the mixture was diluted with water and the product extracted twice with ether. The ether was washed with water, dried (Na_2SO_4) and evaporated. The residue (45 mg.) in acetic acid (1 ml.) was oxidized for 15 min. at about 20° with 20% CrO_3 (0·1 ml.). Water and NaCl (excess) were added and the product was extracted twice with ether. Evaporation of the washed and dried extract left a gum, which was boiled under reflux for 45 min. with water (5 ml.) and 2N-KOH (1 ml.). The cooled mixture was filtered and the filtrate treated with 2N-HCl and NaCl (excess). The amorphous solid was collected, washed and dried by evaporation *in vacuo* with ethanol. The product (23 mg.) was separated on Celite (10 g.) with the system (EC_3) light petroleum (b.p. 80–100°)–water–ethanol (10:1:9, by vol.). After equilibration, the lower (stationary) phase (5 ml.) was put on to the Celite. The separation was carried out exactly as described above for the complex, m.p. 205°. Moving phase (28–58 ml.) eluted a broad band of material (12·2 mg.) giving on paper chromatograms a single spot corresponding to ethyl nordeoxycholate. This material was hydrolysed by warming for 20 min. with ethanol (0·2 ml.) containing 5N-KOH (0·05 ml.). Ethanol was evaporated in N_2 and the residue dissolved in water. The filtered solution was acidified with 2N-HCl and the solid acid was collected, washed and crystallized from acetone, from which it gave large colourless crystals (6 mg.) of m.p. about 140 and 209–211°, not depressed by authentic nordeoxycholic acid (m.p. about 150 and 210–212°) prepared by the method of Riegel *et al.* (1944). A sample of each acid was methylated with diazomethane and the infrared spectra of the products were found to be identical.

Conversion of phocaecholic acid into norchenodeoxycholic acid

Phocaecholic acid (40 mg.) was dissolved in 4 ml. of 0·05M-lead tetra-acetate in acetic acid. After 17·5 hr. at 49°, the mixture was diluted with sodium acetate–KI

reagent (4 ml.) and iodine was titrated with 0·05N-$Na_2S_2O_3$; NaCl (excess) was added and the product extracted twice with ether. The ether was washed with water, dried (Na_2SO_4) and evaporated. The residue was left for 65 hr. in pyridine (1 ml.) with acetic anhydride (1 ml.). The solution was diluted with 2N-HCl and the product recovered with ether as before. Evaporation of the washed and dried ether left a gum which was dissolved in acetic acid (0·5 ml.) and oxidized with 20% CrO_3 (0·2 ml.) at about 20° for 20 min. After treatment with water and NaCl (excess) the product was recovered with ether as before. Evaporation of the washed and dried ether left a residue which was gently warmed and shaken with dilute aqueous $NaHCO_3$. The solution was filtered and the small insoluble residue and filter were washed with water. The combined filtrate and washings were acidified with HCl and the precipitated acid was collected, washed, dissolved in acetone and evaporated to dryness *in vacuo*. The residue was converted into ethyl esters as described above. The partly crystalline esters (26 mg.) were purified by elution from Al_2O_3 (0·4 g.) in a column with benzene (100 ml.). The residue (21 mg.) obtained by evaporating the benzene had an indefinite m.p. and was re-acetylated by dissolution in acetic acid (0·5 ml.) with acetic anhydride (0·1 ml.), followed by treatment with 8·5N-perchloric acid (1 drop). After 20 min. the mixture was diluted and the product was recovered with ether as before. Evaporation of the washed ether left a crystalline residue (20 mg.), which from light petroleum gave colourless prisms, m.p. 128–133°, and separated from aqueous ethanol as long prisms of m.p. 131–133°, not depressed by authentic ethyl diacetyl norchenodeoxycholate and showing the same infrared spectrum as this substance.

Preparation of norchenodeoxycholic acid

To a Grignard reagent made from bromobenzene (1·2 ml.), magnesium (0·25 g.) and ether (25 ml.) was added, with shaking, a solution of methyl chenodeoxycholate (0·25 g.) in benzene (8 ml.). The mixture was heated under reflux for 1 hr. and then poured on to a mixture of 10N-HCl and ice. The product was extracted twice with ether and the extract was washed with water, 2N-KOH and water and was dried (Na_2SO_4). Evaporation left a residue which was suspended in water, through which steam was passed for 40 min. After cooling, the material not volatile in steam was collected and dried by evaporation *in vacuo* with ethanol–benzene. The residue was boiled under reflux for 1 hr. with a mixture of acetic acid (2·5 ml.) and acetic anhydride (1·25 ml.). Part (2 ml.) of this mixture was then distilled off and the remainder was diluted with water and extracted three times with ether. The extract was washed with water, aqueous ammonia and water and dried (Na_2SO_4). Evaporation left a brown gum (0·39 g.) which was dissolved in acetic acid (2 ml.) and oxidized at 50±3° with a solution of CrO_3 (0·3 g.) in 3·3 ml. of water. After 20 min. at 50±3°, the solution was poured into a saturated solution of NaCl and extracted three times with ether. The ether was washed with water, N-KOH and water. The combined alkaline washings were heated at about 80° in a stream of N_2 to expel ether, cooled and treated with 2N-HCl and NaCl (excess). After cooling to about 0°, the white solid precipitate was collected and dried by evaporation *in vacuo* with acetone. The residue (0·11 g.) was esterified with ethanol as described above; the product (98 mg.) gave hydrated needles of m.p. 67–74° from light petroleum–benzene. These (50 mg.) were acetylated for 15 min. in acetic acid (1 ml.) with acetic anhydride (0·2 ml.) and 8·5N-perchloric acid (1 drop). After addition of water (excess) a crystalline solid separated, which was collected, washed and crystallized twice from aqueous ethanol, from which *ethyl diacetyl norchenodeoxycholate* gave long colourless prisms of m.p. 131–134°; $[\alpha]_D^{25}$ +18·7±1° in ethanol (*c*, 1·6) (Found: C, 71·6; H, 9·3; $C_{29}H_{46}O_6$ requires C, 71·0; H, 9·4%). This ester (27 mg.) in ethanol (1 ml.) was warmed for 35 min. under reflux with 5N-KOH (0·1 ml.). Ethanol was removed in N_2 and the residue dissolved in water and treated with 2N-HCl and NaCl (excess). The precipitated acid was collected, washed and crystallized from aqueous ethanol, from which it formed long white needles, which appeared to lose solvent before melting, with decomposition, at 199–202°. This *norchenodeoxycholic acid* had $[\alpha]_D^{23}$ +12·6±1° in ethanol (*c*, 1·6) (Found: C, 71·4; H, 10·0; $C_{23}H_{38}O_4,\frac{1}{2}H_2O$ requires C, 71·3; H, 10·1%).

DISCUSSION

Nomenclature. Hammersten (1909, 1910) described as 'β-phocaecholic acid' a substance shown by Windaus & van Schoor (1928) to be probably (I). This constitution is confirmed by the present work. Hammarsten also found an 'α-phocaecholic acid' of unknown structure. Bergström *et al.* (1959) state that some of Hammarsten's original preparations consist almost entirely of 3α:7α:12α:23-tetrahydroxycholanic acid (IV). The acid (IV) cannot, however, be called 'α-phocaecholic acid', for it is not a cholic (trihydroxycholanic) acid in the sense used until the present time in bile acid nomenclature. It is proposed that Hammarsten's 'β-phocaecholic acid' be called 'phocaecholic acid'. The acid isomeric at C-23 will then be 23-*iso*phocaecholic acid, and can, like Hammarsten's 'β-acid', be eventually named in full as a trihydroxycholanic acid. The 3α:7α:12α:23-tetrahydroxycholanic acid of Bergström *et al.* and of the present work can be so named, or can be called 12α-hydroxyphocaecholic acid if it is shown to have the same configuration at C-23 as phocaecholic acid itself.

Chemical. (i) Cholic acid isomer whose ethyl ester gave peak A (Fig. 1). This substance is not *allo*(5α)cholic acid, but the infrared spectrum (Fig. 2) of its methyl ester shows two bands at about 9·73 and 9·91 μ, very similar in relative intensity to those at about the same wavelengths as are given by methyl *allo*cholate. A constitution which would possibly account for the other properties (specific rotation, behaviour on chromatograms, response to the Hammarsten test) of the new acid is that of 3β:7α:12α-trihydroxy*allo*(5α)-cholanic acid. Alternatively, the new substance may be a trihydroxy derivative of a hitherto undescribed carbon-skeleton isomer of cholanic acid.

(ii) Tetrahydroxy acid whose ethyl ester gave peak B (Fig. 1). This acid is undoubtedly substantially the same as the 'α-phocaecholic acid' of Bergström *et al.* (1959). Their evidence for its constitution as (IV) was based to an important extent on its conversion into norcholic acid. However, the optical rotation given ($[\alpha]_D + 22{\cdot}9°$ in ethanol) does not agree with the proposed structure, and the published melting point (152°) of the derived norcholic acid is not that (about 183°) obtained in this laboratory or published in the literature (Shimizu & Kazuno, 1936; Morsman, Steiger & Reichstein, 1937) for this substance.

The optical rotation ($[\alpha]_D + 20{\cdot}5°$ in ethanol) given by Bergström *et al.* (1959) for 'β-phocaecholic acid' also does not agree with the expected value (given in the present report) and perhaps the rotation figures in the paper of Bergström *et al.* should be discounted. Although the norcholic acid isolated may have contained impurities, as its melting point suggests, the fact that its mass spectrum was identical with that of authentic norcholic acid suggests that it most probably consisted essentially of this compound. From other experience, the writer has reason to believe that contamination with *allo*(5α)norcholic acid would not necessarily be detected in the mass spectrum. Such contamination would probably not be removed by partition chromatography and might have resulted from the presence of some *allo*(5α) acid in the original tetrahydroxycholanic acid. The writer's specimen of this acid might also be similarly contaminated, and such impurities would be sufficient to account for the small differences in infrared spectra between the Swedish material and that described here. Another source of differences might be the presence of different proportions of optical enantiomers at C-23. In summary, the evidence is strongly in favour of the view that the 'α-acid' of Bergström *et al.* (1959) is, in fact, 3α:7α:12α:23-tetrahydroxycholanic acid (IV), and that this is also the constitution of the corresponding acids from leopard seal and the snake bile now examined. In none of the acids hydroxylated at C-23 described has the optical configuration been elucidated, and it cannot even be confidently stated that they are, in fact, specimens of single optical enantiomers at this centre.

Biological. The two snakes of the genus *Bitis* comprise the only animals, except Pinnipedia, so far examined which have been shown to have bile acids hydroxylated at C-23. This chemical feature seems, by its distribution, to be a true species character and not an artifact of intestinal micro-organisms. If this is so, the livers of the species concerned presumably contain a C-23-hydroxylating-enzyme system, the presence of which should be detectable by direct experiment.

Apart from C-23 hydroxylation, the bile salts of the *Bitis* snakes and of the leopard seal are not more closely similar than those of other unrelated animals. In this seal, the chief bile acid is phocaecholic acid, which may, like chenodeoxycholic acid, be a 'primary' bile acid, made by the liver from cholesterol: 3α:7α:12α:23-tetrahydroxycholanic acid is a minor constituent. On the other hand, bitocholic acid, lacking a hydroxyl group at C-7, is, like deoxycholic acid, likely to be an artifact, made during the enterohepatic circulation by intestinal micro-organisms from 3α:7α:12α:23-tetrahydroxycholanic acid, which in these snakes is present in major amounts.

The *allo*cholic acid found in leopard-seal bile is possibly derived from fishes or penguins eaten, for these are likely to have contained this substance (Anderson, Haslewood & Wootton, 1957; Anderson & Haslewood, 1960). A discussion of the possible biological implications of the presence of the *iso*-cholic acid now newly found in snake bile must await the elucidation of its chemical constitution. It seems unlikely to be a dietary artifact, for Dr Osman Hill, who supplied the bile, states that the snakes had been fed on newborn rats and mice and chicks, the bile of which is not known to contain such an acid.

If the peculiar chemical nature of the bile salts of the Gaboon viper and puff adder is shared only by the few other snakes of the genus *Bitis*, consideration might be given to the more definite separation of this group of reptiles from the remainder of the Viperidae.

SUMMARY

1. Bile salts of the leopard seal, *Hydrurga leptonyx*, and of the Californian sealion, *Zalophus californianus*, have been shown to contain the 'β-phocaecholic acid' of Hammarsten (1909, 1910), which it is now proposed to call 'phocaecholic acid'. Leopard-seal bile was shown to contain also *allo*(5α)cholic acid and the 3α:7α:12α:23-tetrahydroxycholanic acid identified by Bergström *et al.* (1959) as Hammarsten's (1909, 1910) 'α-phocaecholic acid'. It is suggested that this name is inapplicable, as the substance is not a 'cholic' (trihydroxycholanic) acid.

2. Bile salts of the Gaboon viper, *Bitis gabonica*, and puff adder, *Bitis arietans*, contained a new acid, called 'bitocholic acid', an unidentified isomer of cholic acid and the same 3α:7α:12α:23-tetrahydroxycholanic acid as was found in seal bile.

3. Bitocholic acid was shown by its properties and by degradation to the known nordeoxycholic acid (3α:12α-dihydroxynorcholanic acid) to be 3α:12α:23-trihydroxycholanic acid.

4. Phocaecholic acid was degraded to norchenodeoxycholic acid (3α:7α-dihydroxynorcholanic acid), also prepared by Wieland–Barbier degradation from chenodeoxycholic acid. This work confirms the constitution suggested for phocaecholic acid by Windaus & van Schoor (1928).

5. In none of the C-23-hydroxylated bile acids described could the optical configuration at C-23 be determined by measurements of $[\alpha]_D$.

6. Although snakes of the genus *Bitis* contain C-23-hydroxylated bile acids, previously thought to be confined to the Pinnipedia, the types of bile acids present do not indicate a very close similarity between the bile salts of these snakes and those of Pinnipedia.

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REFERENCES

Anderson, I. G. & Haslewood, G. A. D. (1960). *Biochem. J.* **74**, 37P.
Anderson, I. G., Haslewood, G. A. D. & Wootton, I. D. P. (1957). *Biochem. J.* **67**, 323.
Baer, E. (1940). *J. Amer. chem. Soc.* **62**, 1597.
Bergström, S., Krabisch, L. & Lindeberg, U. G. (1959). *Acta Soc. Med. Upsalien.* **64**, 160.
Bush, I. E. (1952). *Biochem. J.* **50**, 370.
Hammarsten, O. (1909). *Hoppe-Seyl. Z.* **61**, 454.
Hammarsten, O. (1910). *Hoppe-Seyl. Z.* **68**, 109.
Haslewood, G. A. D. (1956). *Biochem. J.* **62**, 637.
Haslewood, G. A. D. & Ogan, A. U. (1959). *Biochem. J.* **73**, 142.
Haslewood, G. A. D. & Sjövall, J. (1954). *Biochem. J.* **57**, 126.
Haslewood, G. A. D. & Wootton, V. (1950). *Biochem. J.* **47**, 584.
Haslewood, G. A. D. & Wootton, V. M. (1951). *Biochem. J.* **49**, 67.
Morsman, H., Steiger, M. & Reichstein, T. (1937). *Helv. chim. acta*, **20**, 3.
Riegel, B., Moffett, R. B. & McIntosh, A. V. (1944). *Org. Synth.* **24**, 38.
Shimizu, T. & Kazuno, T. (1936). *Hoppe-Seyl. Z.* **244**, 167.
Windaus, A. & van Schoor, A. (1928). *Hoppe-Seyl. Z.* **173**, 312.

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The Breakdown of Chlorophyll by Chlorophyllase

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The first stage in the enzymic breakdown of chlorophyll *in vivo* may be the removal of the phytol side chain; chlorophyllase, the enzyme which *in vitro* catalyses its removal and attachment, has been found in most plants. The enzyme was discovered by Willstätter & Stoll (1910), and Mayer (1930) made an extensive survey of chlorophyllase activity in different species and investigated some properties of the enzyme. Krossing (1940) found that it was in the chloroplast fraction of leaves. Chlorophyllase action *in vitro* usually requires the presence of a solvent such as acetone (40–70 %, w/v) or methanol (70–80 %), but Weast & Mackinney (1940) found that in a few species at some seasons of the year there was no hydrolysis in ethanol, though an enzyme system active in water at 70° was present. Peterson & McKinney (1938) investigated the effect of mosaic viruses on the chlorophyllase activity and chlorophyll content of tobacco leaves.

Dried leaves have frequently been used as a source of enzyme for preparing chlorophyllides from chlorophyll. Fischer & Lambrecht (1938) used a leaf-meal preparation for studying the specificity of chlorophyllase action with various chlorophyll derivatives.

Mayer (1930) found that little or no chlorophyllase activity was extracted from leaf powders by water, aqueous buffer solutions or glycerol. Willstätter & Stoll (1910) obtained a trace of soluble chlorophyllase by subjecting macerated fresh leaves to 250 atmospheres pressure. Ardao & Vennesland (1960) found that a soluble chlorophyll–lipoprotein complex obtained when spinach chloroplasts are treated with digitonin had chlorophyllase activity.

The present paper is concerned with the preparation of soluble chlorophyllase, its partial purification and some of its properties.

MATERIAL AND METHODS

Plant material

Sugar beet (*Beta vulgaris* var. *saccharifera*) leaves were obtained from experimental plots on Rothamsted Farm. The leaves could be kept at 0° in polythene bags for up to a