Comparative Studies of 'Bile Salts'

15. THE NATURAL OCCURRENCE AND PREPARATION OF ALLOCHOLIC ACID*

BY I. G. ANDERSON AND G. A. D. HASLEWOOD
Guy's Hospital Medical School, London, S.E. 1

(*Received* 7 *May* 1962)

From the bile of the 'Gigi' fish and, later, from other species of teleosts Ohta (1939) obtained an acid to which he assigned the formula $C_{27}H_{46}O_6$ and the name 'tetrahydroxynorsterocholanic acid'. Ohta (1939) and Isaka (1940) reported experiments which indicated that 'Ohta's acid' was a 3α,6α,12,*x*-tetrahydroxy acid, which could be oxidized to a 3α,6α,12-trihydroxycholanic acid ('isocholic acid'). 3α,6α,12α-Trihydroxycholanic acid was prepared by Takeda & Igarashi (1956, 1959) and also by Haslewood (1958) and has properties quite different from those of Ohta's acid or of 'isocholic acid'. When Haslewood & Wootton (1956) examined the infrared spectrum of a Japanese sample of Ohta's acid, they found that it resembled that of methyl cholate (methyl 3α,7α,12α-trihydroxycholanate). Anderson, Haslewood & Wootton (1957) isolated Ohta's acid, identical with a Japanese specimen, from the bile of the king penguin and concluded, by analysis of its ethyl ester, that it was probably isomeric with cholic acid, $C_{24}H_{40}O_5$.

Professor K. Yamasaki, who had previously obtained the acid from chicken bile (Yamasaki, 1951), informed us that he had confirmed the findings of Ohta (1939) that it could be converted into a mixture of cholanic (5β-H) and allocholanic (5α-H) acids, $C_{24}H_{40}O_2$. It was this experiment which had led Ohta to infer that his new acid was substituted by a hydroxyl group at C-6, for the derived ketone would be expected to give allo derivatives on treatment with alkali, as Ohta found.

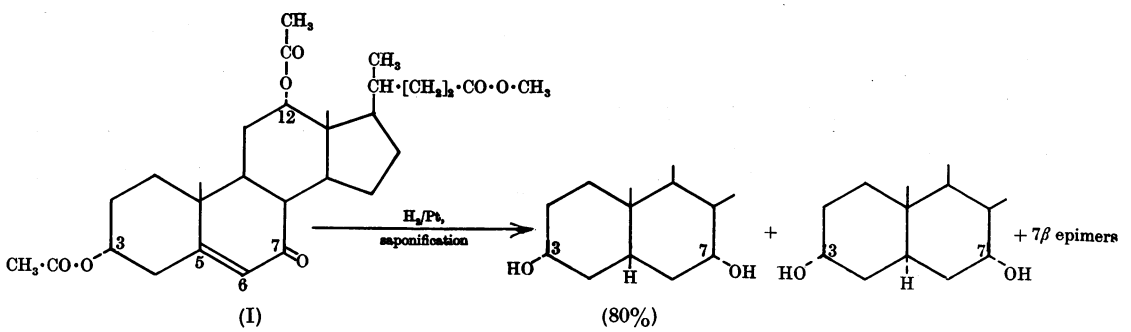
* Part 14: Bridgwater, Briggs & Haslewood (1962).

It occurred to us that another explanation might be that Ohta's acid itself was or contained an allo compound, and we made material believed to be allocholic acid, and identical with the best available natural samples, by hydrogenation of methyl 3α,12α-diacetoxy-7-oxochol-5-enate (I) (Anderson & Haslewood, 1960); this work is here described in full.

What seemed to be another allo isomer of cholic acid was isolated from the bile of snakes of the genus *Bitis* (Haslewood, 1961). Meanwhile, we had failed to convince ourselves that we could fully separate allocholic acid from cholic acid after the hydrogenation mentioned above, and we thought it essential to devise a method for preparing allocholic acid which did not involve this separation. Such a method was found (Anderson & Haslewood, 1961) and is now described; it gave allocholic acid identical with the supposed 'isomer' from snake bile.

RESULTS

Methyl 3α,12α-diacetoxy-7-oxochol-5-enate (I) was made by an improvement of the method of Takeda & Komeno (1954, 1957). It rapidly took up hydrogen with Adam's catalyst in acid solution, and the saturated mixed esters after saponification and re-esterification could be recrystallized to give, in about 10% yield, material of m.p. about 208°, not depressed by the methyl ester of Ohta's acid from natural sources and showing the same $[\alpha]_D$ (about +28° in ethanol) and infrared spectrum as this material. The ethyl ester had corresponding properties, but the free acid did not crystallize.



(I) (80%)

The chief products of the process were cholic acid derivatives, in a yield probably of at least 80%. Paper chromatography of the methyl esters showed a major spot with the R_F of methyl cholate and a minor spot with the R_F of methyl 3α,7β,12α-trihydroxycholanate. The latter spot was probably given by a mixture of this substance and its allo isomer.

When 3α,6α-dihydroxy-7-oxocholanic acid or its esters are heated in ethanol with aqueous alkali, there is formed an acid ('acid *A*' of Haslewood, 1956, 1958) to which Takeda, Komeno & Igarashi (1954) tentatively assigned the formula 3α,7β-dihydroxy-6-oxoallocholanic acid (III). Ziegler (1956) found some evidence in support of this formula, and we have now prepared (III) from ethyl 3α-acetoxy-6-oxoallocholanate (II), as shown. As reported (Anderson & Haslewood, 1961) acid (III) was converted by treatment with Raney nickel of the thioketal of its methyl ester into methyl 3α-hydroxyallocholanate; we now describe other products, the $[\alpha]_D$ of one of which did not agree with the expected constitution, i.e. 3α,7β-dihydroxyallocholanic acid.

When either methyl 3α,6α,12α-triacetoxy-7-oxocholanate (IV) or ethyl 3α,12α-diacetoxy-6α-bromo-7-oxocholanate was boiled in dioxan with aqueous sodium hydroxide, the only crystalline material isolated was 3α,7β,12α-trihydroxy-6-oxoallocholanic acid (V), agreeing in properties (except for $[\alpha]_D$) with an acid described by Takeda *et al.* (1954). The crystalline thioketal of the methyl ester of (V) was boiled in ethanol with Raney nickel and the chief acid obtained, after hydrolysis, proved to be 3α,7β,12α-trihydroxyallocholanic acid (VI), although there were minor amounts of other substances. Partial oxidation of (VI) with potassium chromate–acetic acid–sodium acetate gave a good yield of 3α,7β-dihydroxy-12-oxoallocholanic acid (VII). Chromic oxidation of (VI) gave dehydroallocholic acid (3,7,12-trioxoallocholanic acid) (IX), (hydrate) m.p. 232°, $[\alpha]_D + 28°$ (in ethanol).

For the preparation of allocholic acid, it was convenient to oxidize the ethyl ester of (VI) in methanol with *N*-bromosuccinimide corresponding to 2·25 g.atoms of available oxygen: the product was a mixture which was reduced with sodium borohydride (tetrahydroborate), or better, with hydrogen–platinum in acid solution. The resulting ethyl esters could be easily separated on Celite, giving, in a yield of about 22% and as the chief crystalline product, a substance (ethyl allocholate) identical with the isomer of ethyl cholate from snake bile (Haslewood, 1961). Saponification of this yielded allocholic acid [3α,7α,12α-trihydroxyallo(5α)cholanic acid] (VIII), m.p. (from acetone) 241°; $[\alpha]_D + 23°$; the infrared spectrum is shown in Fig. 1A. The remainder of the product from the reactions (VI) → (VIII) consisted of less polar material, apparently products of dehydration during the hydrogenation, and also contained ethyl 3α,7β,12α-trihydroxyallocholanate.

Mixtures (2:1, w/w) of methyl allocholate and ethyl allocholate with the corresponding cholic acid esters closely resembled esters of Ohta's acid.

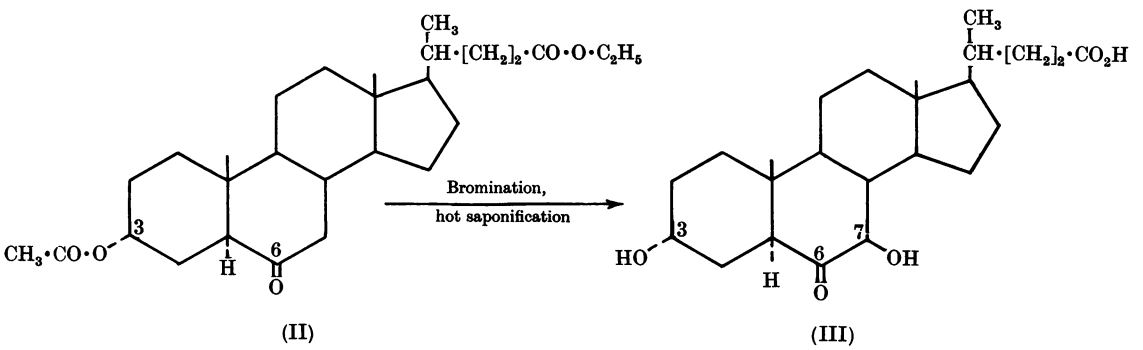
Allocholic acid, like cholic acid, gave an intense purple colour in the Hammarsten (hydrochloric acid) test, whereas 3α,7β,12α-trihydroxycholanic acid and its allo isomer gave a clear yellow colour. Prolonged chromatography on paper showed that allocholic acid moved very slightly faster than cholic acid in the solvent systems used.

EXPERIMENTAL

General. Details, where applicable, were as given by Bridgwater, Briggs & Haslewood (1962). Raney nickel was as prepared by Haslewood (1958), and esterification was done as by Anderson *et al.* (1957).

Hydrogenation of methyl 3α,12α-diacetoxy-7-oxochol-5-enate (I)

Methyl 6α-bromo-3α,12α-diacetoxy-7-oxocholanate (*cf. Takeda & Komeno*, 1954, 1957). Methyl 3α,12α-diacetoxy-7-oxocholanate (10 g.) in acetic acid (10 ml.) was treated with a solution of bromine (1·2 ml.) in acetic acid (10 ml.). After not less than 4 hr., the mixture was poured into water and the solid product collected, washed with water and dissolved in ether. The ether was dried (over Na_2SO_4),

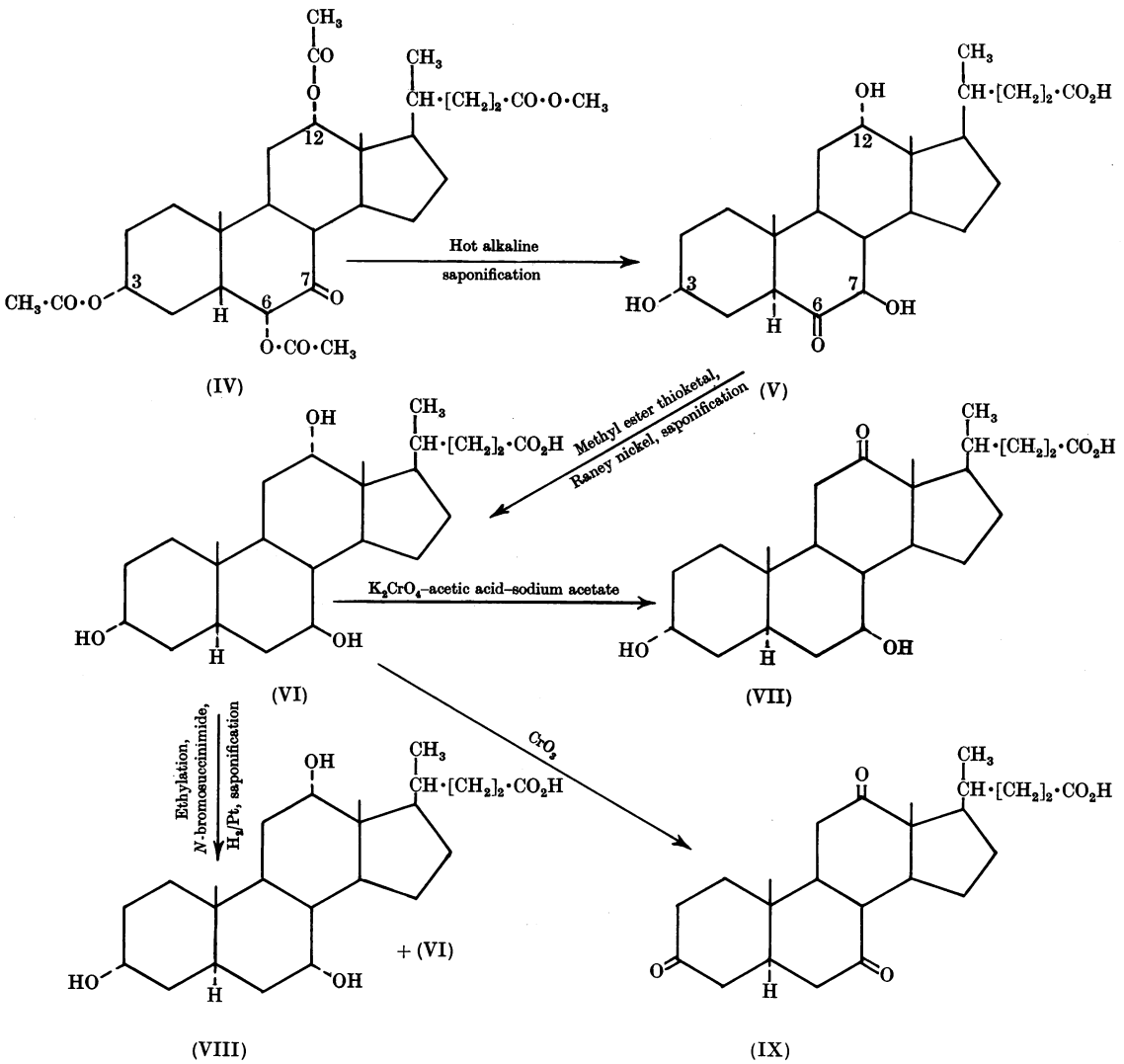


(II) (III)

filtered, evaporated to about 200 ml. and cooled to 0°. The crystalline precipitate was collected and washed with cold ether. The yield was 7·5 g. of crystals, m.p. about 165°.

Methyl 3α,12α-*diacetoxy*-7-*oxochol*-5-*enate* (I). The above bromo compound (3 g.) was added to a solution of silver nitrate (4 g.) in pyridine (20 ml.). Dry nitrogen was gently bubbled through the solution as it was boiled under reflux for 8·5 hr. The product was poured into N-HNO_3 (excess) and this was extracted with ether (3 ×). The ether was washed with water, aqueous NH_3 and water, and dried (over Na_2SO_4). The residue left after evaporation of the filtered ether was crystallized at 0° from a little fresh ether, collected and washed with cold ether. The yield of (I) was about 1 g. Recrystallization from aq. 50% (v/v) methanol gave white needles, m.p. 214–218° (decomp.).

Esters of Ohta's acid from (I). Substance (I) (0·5 g.) was dissolved in methanol (40 ml.) containing 10N-H_2SO_4 (8 drops). Adams catalyst (50 mg. of PtO_2) was added and the mixture stirred in a hydrogen atmosphere. The uptake of hydrogen was quantitative for $2H_2$/molecule after 15 min., after which it ceased. The product was decanted into water and extracted with ether. The washed and dried (over Na_2SO_4) ether was evaporated, leaving a colourless gum which was heated under reflux with ethanol (10 ml.) and 5N-KOH (2 ml.) for 1 hr. Evaporation of ethanol left a residue which was precipitated from water with 2N-HCl and NaCl (excess). After cooling at 0°, the solid acid was collected, washed with water, dissolved in methanol and esterified with diazomethane. An ethereal solution of the product was washed with water and evaporated. With fresh ether (20 ml.) the residue gave long needles (73 mg.), m.p. 206–209°, which had the same infrared spectrum as and did not depress the m.p. (204–209°) of a specimen of the methyl ester of Ohta's acid, supplied by Professor K. Yamasaki. The derived acid did not crystallize; it had $[\alpha]_D^{22} + 28 \pm 1°$ (*c* 2·1 in ethanol). The corresponding ethyl



(IV) (V) (VI) (VII) (VIII) (IX)

ester, from benzene, gave fine white needles, m.p. 202–204°, not depressed by the ethyl ester of Ohta's acid. Evaporation of the original ethereal mother liquors gave material (0·31 g.) which had $[\alpha]_D^{21}+37\pm1°$ (*c* 2·7 in ethanol) and consisted chiefly of methyl cholate. Paper chromatography in 'system G_3' of Haslewood & Sjövall (1954) showed that (*a*) the methyl ester m.p. 206–209° had the same R_F as methyl cholate and (*b*) the material from the ether liquors gave, in addition, a spot with the R_F of methyl 3α,7β,12α-trihydroxycholanate, made from a specimen of the corresponding acid supplied by Professor S. Bergström. All attempts to separate higher-melting-point material from the above methyl or ethyl esters failed.

Compounds from 3α,7β-*dihydroxy*-6-*oxoallocholanic acid* (III)

Compound (III) *from ethyl* 3α-*acetoxy*-6-*oxoallocholanate* (II). Ethyl 3α-hydroxy-6-oxoallocholanate (4 g., from pig bile; Haslewood, 1956) was acetylated by the perchloric acid method in the usual way and the product, recovered after extraction with ether, was dissolved in acetic acid (20 ml.) and treated with 20 ml. of a solution of bromine (0·6 ml.) in acetic acid (25 ml.). After 16 hr., the mixture was poured into water and the brown solid was collected, washed with water and boiled under reflux for 75 min. with dioxan (40 ml.), water (40 ml.) and 5N-KOH (8 ml.). Dioxan was removed *in vacuo* and the aqueous residue treated with 2N-HCl and NaCl (excess). After cooling at 0°, the solid acid was collected, washed with water and dissolved in ethyl acetate. Evaporation of the filtered solution left a residue which, with acetone, gave crystals. These, after refrigeration, were collected, washed with cold acetone and dissolved in ethanol. Water was added and the solution was heated on a water bath and evaporated under a nitrogen jet until crystallization began. After cooling at 0°, the product was collected and washed with aq. 30% (v/v) ethanol. It consisted of long white needles (1·05 g.), m.p. 232–234° (decomp.), of 3α,7β-dihydroxy-6-oxoallocholanic acid (III), identical (mixed m.p.; infrared spectrum) with 'acid *A*' from hyocholic acid (Haslewood, 1958).

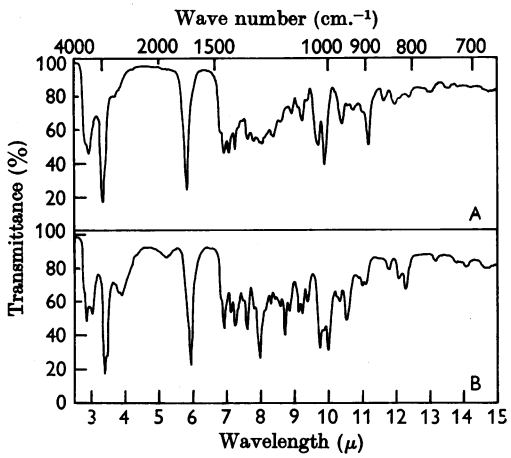


Fig. 1. Infrared spectra in potassium bromide. A, 3α,7α,12α-Trihydroxyallocholanic acid (allocholic acid); B, 3α,7β,12α-trihydroxyallocholanic acid.

Methyl 3α,7β-*dihydroxy*-6-*oxoallocholanate thioketal*. 3α,7β-Dihydroxy-6-oxoallocholanic acid (III; 0·5 g.) was suspended in methanol and esterified with diazomethane; an ethereal solution of the product was washed with water and evaporated. The resultant gum was mixed with ethane-1,2-dithiol (1·5 ml.) and boron trifluoride etherate (0·15 ml.) when crystallization at once began. After 70 hr., the mixture was dissolved in ether (25 ml.) and the solution washed with 2N-NaOH (3 × 10 ml.) and water until alkali-free, then dried (over Na_2SO_4) and evaporated. The residue was a solid (475 mg.) which, from acetone, gave colourless prisms of *methyl* 3α,7β-*dihydroxy*-6-*oxoallocholanate thioketal*, m.p. 181–183°, unaltered by recrystallization; $[\alpha]_D^{23}+48\pm1°$ (*c* 1·5 in ethanol) (Found: C, 65·8; H, 9·0. $C_{27}H_{44}O_4S_2$ requires C, 65·3; H, 8·9%).

Methyl 3α-*hydroxyallocholanate*. The above thioketal (0·1 g.) was converted by hydrolysis with ethanolic KOH into the corresponding acid, which was remethylated and then boiled under reflux for 19 hr. with ethanol (10 ml.) and Raney nickel (from 1·5 g. of alloy). Evaporation of the filtered mixture left a gum (86 mg.) which with light petroleum (b.p. 40–60°) gave a solid (76 mg.) which was crystallized twice from methanol. The final product (20 mg.), m.p. 161–164°, had the properties of methyl 3α-hydroxyallocholanate (Found: C, 76·7; H, 10·5. Calc. for $C_{25}H_{42}O_3$: C, 76·9; H, 10·8%).

Other products from the thioketal from (III). In another experiment, the crude solid thioketal (475 mg.) was boiled under reflux for 17 hr. with ethanol (75 ml.) and Raney nickel (from 9·0 g. of alloy). Evaporation of the filtered mixture left a gum (323 mg.) which, in benzene, was separated on Al_2O_3 (neutralized, 9·7 g.). Elution was as follows: fraction I (solvent, 35 ml. of benzene), 2 mg. eluted; fraction II (solvent, 590 ml. of ether), 234 mg. eluted; fraction III (solvent, 200 ml. of ethyl acetate), 79 mg. eluted; fraction IV (solvent, 100 ml. of ethanol), 0 mg. eluted; the total eluted was 315 mg. Fraction II was hydrolysed with ethanolic NaOH to the corresponding acid, which, after recovery in the usual way and being dried by evaporation with methanol, crystallized from ethyl acetate (35 ml.) in fine needles (85 mg.). These, after recrystallization, had m.p. 240–241°; $[\alpha]_D^{22}+60\pm1°$ (*c* 1·28 in ethanol) (Found: C, 73·0; H, 10·2. $C_{24}H_{40}O_4$ requires C, 73·4; H, 10·3%). In 'system A' of Bush (1952), an ethylated sample of this acid showed two spots on paper chromatograms.

Fraction III was similarly hydrolysed, but gave an acid which separated from ethyl acetate as a gel (33 mg.). The methyl ester crystallized on standing with light petroleum (b.p. 40–60°), and recrystallization from aqueous methanol gave needles (12 mg.), m.p. 156–158°; $[\alpha]_D^{24}+58\pm1°$ (*c* 0·72 in ethanol) (Found: C, 73·4; H, 10·5. $C_{25}H_{42}O_4$ requires C, 73·9; H, 10·4%). In 'system A' of Bush (1952), this substance ran as a single spot with an R_F close to that of methyl 3α,7β-dihydroxycholanate.

3α,7β-12α-*Trihydroxy*-6-*oxoallocholanic acid* (V) *and products derived from it*

3α,7β,12α-*Trihydroxy*-6-*oxoallocholanic acid*. Ethyl 3α,-12α-diacetoxy-6α-bromo-7-oxocholanate (3 g.; Haslewood, 1958) was boiled for 1 hr. under reflux with dioxan (40 ml.) and N-NaOH (40 ml.). Dioxan was removed *in vacuo* and

the acid precipitated with 2N-HCl and NaCl (excess). After cooling at 0°, the solid product was collected, washed with water, and evaporated with acetone–ethanol to dryness. The residue was left with a little ethyl acetate and, after refrigeration, the crystalline product was collected and washed with this solvent. A solution of the product in aqueous ethanol was filtered and the filtrate evaporated on a water bath in a nitrogen jet until crystallization began. After refrigeration, the crystals were collected and washed with aq. 30% (v/v) ethanol. The yield was 0·66 g. of 3α,7β,12α-trihydroxy-6-oxoallocholanic acid (V), m.p. 218–220° (decomp.); $[\alpha]_D^{21}+50\pm2°$ (*c* 1·5 in ethanol) (Found: C, 68·5; H, 9·3. Calc. for $C_{24}H_{38}O_6$: C, 68·25; H, 9·1%). The same acid was similarly obtained from methyl 3α,6α,12α-triacetoxy-7-oxocholanate (IV).

Methyl 3α,7β,12α-*trihydroxy*-6-*oxoallocholanate thioketal*. 3α,7β,12α-Trihydroxy-6-oxoallocholanic acid (V; 1 g.) was suspended in methanol and esterified with diazomethane; an ethereal solution of the product was washed with water and evaporated. The resultant gum was mixed with ethane-1,2-dithiol (3 ml.) and boron trifluoride etherate (0·3 ml.), and the mixture shaken intermittently during 1·5 hr., when it became homogeneous. After standing for 17 hr., the product was dissolved in ether (30 ml.) and the solution washed with 2N-NaOH (3 × 20 ml.), and water until alkali-free, then dried (over Na_2SO_4) and evaporated, to yield a solid residue. The product was left in contact with ether–light petroleum (b.p. 40–60°) (3:2, v/v) for 1 hr. at 0°, and the supernatant liquid then decanted. Repetition of this process left a residue which, after recrystallization from acetone, gave *methyl* 3α,7β,12α-*trihydroxy*-6-*oxoallocholanate thioketal*, m.p. 228–229°; $[\alpha]_D^{24}+63\pm2°$ (Found: C, 63·7; H, 9·2. $C_{27}H_{44}O_5S_2$ requires C, 63·2; H, 8·6%).

3α,7β-12α-*Trihydroxyallocholanic acid* (VI). A solution of the above thioketal (0·7 g.) in ethanol (70 ml.) was boiled gently under reflux for 16 hr. with Raney nickel (from 10·5 g. of alloy). Evaporation of the filtered product left a gum [0·6 g., giving in 'system B_3' of Bush (1952) a spot on paper corresponding to methyl 3α,7β-12α-trihydroxycholanate] which was boiled in ethanol (7 ml.) with 5N-KOH (1 ml.) for 40 min. Ethanol was removed by evaporation and the acid precipitated with 2N-HCl and NaCl (excess). After cooling at 0°, the solid product was collected, washed with water and dissolved in ethanol. Evaporation of the filtered solution left a residue which from ethyl acetate gave (perhaps solvated) crystals of 3α,7β-12α-*trihydroxyallocholanic acid* (VI), m.p. 252–253°; $[\alpha]_D^{22}+61\pm1°$ (*c* 1·2 in ethanol); the Hammarsten (HCl) test gave a yellow colour; the infrared spectrum is shown in Fig. 1B (Found: C, 69·9; H, 9·5. $C_{24}H_{40}O_5$ requires C, 70·6; H, 9·9%). This compound was ethylated and the ester (40 mg.) separated on Celite (10 g.) in 'system EC_1' of Haslewood (1961). The effluent (44–122 ml.) contained ethyl 3α,7β,12α-trihydroxyallocholanate (32·3 mg.) which from light petroleum (b.p. 40–60°) ether gave (probably solvated) needles, m.p. 157–161° (Found, after drying at 80° *in vacuo*: C, 71·7; H, 10·2. $C_{26}H_{44}O_5$ requires C, 71·6; H, 10·1%). The acid from this ester had m.p. 259–261°.

3,7,12-*Trioxoallocholanic acid* (*dehydroallocholic acid*). The above acid (VI; 50 mg.) in acetic acid (2·5 ml.) was treated with 20% CrO_3 (0·3 ml.), with cooling. After 10 min. at about 20°, water was added. The solution at first remained clear, but soon small needles separated. After refrigeration, these were collected and washed with water. The yield was 44 mg. of crystals, m.p. 227–235°. Recrystallization from aqueous ethanol gave fine white needles of 3,7,12-*trioxoallocholanic acid* (hydrate), m.p. 229–232° (decomp.); $[\alpha]_D^{22}+28\pm1°$ (*c* 1·5 in ethanol) (Found: C, 68·7; H, 8·5. $C_{24}H_{34}O_5,H_2O$ requires C, 68·6; H, 8·6%). Ethylation of this, in the usual way, gave *ethyl* 3,7,12-*trioxoallocholanate*, crystallizing from light petroleum (b.p. 40–60°)–benzene as long white prisms, m.p. 164–165° (Found: C, 72·1; H, 8·6. $C_{26}H_{38}O_5$ requires C, 72·6; H, 8·9%).

3α,7β-*Dihydroxy*-12-*oxoallocholanic acid* (VII). 3α,7β,12α-Trihydroxyallocholanic acid (50 mg.) was dissolved in acetic acid (2 ml.), together with crystalline sodium acetate trihydrate (300 mg.). Then 0·05 ml. of a potassium chromate solution (31·7 g./100 ml. in water) was added and the mixture gently shaken until all had dissolved. After 24 hr. water and NaCl (excess) were added. The crystalline precipitate (40 mg.) was collected and recrystallized from aqueous ethanol, from which it gave colourless needles of 3α,7β-*dihydroxy*-12-*oxallocholanic acid* (VII), m.p. 247–248°; $[\alpha]_D^{23}+94\pm1°$ (*c* 0·70 in ethanol) (Found: C, 70·4; H, 9·8. $C_{24}H_{38}O_5$ requires C, 70·9; H, 9·4%).

3α,7α,12α-*Trihydroxyallocholanic acid* (*allocholic acid* (VIII). 3α,7β,12α-Trihydroxyallocholanic acid (100 mg.) was converted into the ethyl ester, and this was dissolved in methanol (3 ml.) and the solution treated with *N*-bromosuccinimide (99 mg.; equivalent to 2·25 g.atoms of available oxygen). Solution was effected by shaking, and the mixture left at room temperature for 17 hr. Water (6 ml.) was added and methanol removed at room temperture in a jet of nitrogen. The product was extracted with ether (3 ×), and the ether washed with water, dried (over Na_2SO_4) and evaporated. The residue (101 mg.) in methanol (10 ml.) and 10N-H_2SO_4 (0·4 ml.) was stirred in an atmosphere of hydrogen for 2 hr., after the addition of Adam's catalyst (81 mg. of PtO_2). After dilution with water, the product was removed with ether, and the ether washed with water, aqueous $NaHCO_3$ and water, dried (over Na_2SO_4) and evaporated. The partially crystalline residue (79 mg.) was separated on Celite (10 g.) in 'system EC_1' of Haslewood (1961). Elution of the principal fractions was as follows: fraction I (20–30 ml. of effluent), 22·9 mg. eluted; fraction II (32–50 ml. of effluent), 23·4 mg. eluted; fraction III (64–98 ml. of effluent), 10·3 mg. eluted; fraction IV (134–164 ml. of effluent), 5·9 mg. eluted. Fraction I was a gum, fraction II consisted almost entirely of ethyl allocholate (identical with the 'peak A' substance similarly obtained by Haslewood, 1961), fraction III was ethyl 3α,7β,12α-trihydroxyallocholanate and fraction IV was a mixture not further investigated.

Attempts were made to separate fraction I (53 mg.) on Celite (10 g.) in light petroleum (b.p. 80–100°)–ethanol–water (7:5:2, by vol.). The moving (light petroleum) phase (44–60 ml.) eluted a crystalline fraction (7 mg.) which on hydrolysis gave an acid that, with ethyl acetate, formed small needles, m.p. 202–205° (decomp.). However, paper chromatography of a methylated sample of this substance showed that it was probably still impure, although most of the ester had about the same R_F as methyl deoxycholate (methyl 3α,12α-dihydroxycholanate).

Combined fraction II (69 mg.) was hydrolysed by boiling it for 20 min. in ethanol (2 ml.) with 5N-KOH (0·2 ml.). The ethanol was evaporated off and the acid precipitated with 2N-HCl and NaCl (excess). After refrigeration, it was

collected, washed with water and dissolved in ethanol. Evaporation of the filtered solution left a residue which on standing with acetone became long colourless prisms (50 mg.). These lost solvent on heating, and melted at 239–241°. This 3α,7α,12α-*trihydroxyallocholanic acid* (*allocholic acid*) (VIII) apparently contained acetone; it had $[\alpha]_D^{22}+23\pm1°$ (*c* 1·2 in ethanol); the Hammarsten test gave a purple colour; the infrared spectrum is shown in Fig. 1A (Found: C, 69·4; H, 9·9. $C_{24}H_{40}O_5,CH_3\cdot CO\cdot CH_3$ requires C, 69·5; H, 9·9%). The ethyl ester of this substance has been described (Haslewood, 1961) and the *methyl ester*, made with diazomethane, did not readily crystallize from solvents, but on heating became needles (which melted at about 225°) of *methyl allocholate* (Found: C, 70·9; H, 10·45. $C_{25}H_{42}O_5$ requires C, 71·05; H, 10·0%). After descending chromatography (by Miss J. Head) for 51 hr. on paper, with dibutyl ether–aq. 20% (v/v) acetic acid (1:1, v/v), it was noted that allocholic acid moved slightly faster than cholic acid, although complete separation did not occur.

Esters of Ohta's acid. Methyl (or ethyl) allocholate (2 mg.) and methyl (or ethyl) cholate (1 mg.) were weighed out together and evaporated in methanol. The residues formed gels from solvents, but on heating became crystalline. The mixed methyl esters melted at 208–210° and the mixed ethyl esters at 201–204°. In each case, the infrared spectra were identical with those given by the methyl (or ethyl) esters of Ohta's acid.

DISCUSSION

Chemical. No authentic allocholanic acids hydroxylated at C-7 or C-12 are known, and the opinion that (VIII) is allocholic acid must be substantiated.

The mixtures, identical with methylated (or ethylated) Ohta's acid, finally obtained from (I) could have contained only esters of cholic acid, allocholic acid, or their C-7β epimers. These mixtures showed on paper chromatograms a single spot, with the R_F of methyl (or ethyl) cholate. Esters of 3α,7β,12α-trihydroxycholanic acid and of what we believe to be 3α,7β,12α-trihydroxyallocholanic acid (VI) ran at about equal rates on paper, and readily separated from those of cholic acid in the solvent systems used. Hence the Ohta's acid esters could not have contained substantial amounts of C-7β-hydroxylated substances, and must therefore have consisted of mixtures of esters of cholic acid and allocholic acid. The fact that these mixtures could be simulated by mixing esters of pure (VIII) and of cholic acid strongly supports the view that (VIII) is allocholic acid.

The sequence (II) → (III) proves that (III) ('acid *A*') is an allo acid and this is confirmed by the preparation of methyl 3α-hydroxyallocholanate from it.

Substance (V) showed the properties expected of '12α-hydroxy-(III)', and the formulae of (VI) and (VII) are based on the belief that (V) has indeed this structure. The structure for (VIII) has, as described above, independent support; this is fortunate, for the thioketal–nickel reduction can lead to unexpected results (see, e.g., Hsia *et al.* 1960). Finally, the ethyl ester of (IX) had the properties deduced for a second component (other than dehydrocholic acid) obtained by partial separation of the chromic oxidation product of an ethylated mixture rich in Ohta's acid from king-penguin bile (Anderson *et al.* 1957). We think that all the above considerations, taken together, leave no reasonable doubt that (VIII) is allocholic acid. The quantitative results of hydrogenating (I) are in accordance with those expected from Fieser & Fieser (1959).

Substance (VII) showed the high $[M]_D$ expected of such a ketone, and irreconcilable with the expected $[M]_D$ of 3α,12α-dihydroxy-7-oxo- or 7β,12α-dihydroxy-3-oxo-allocholanic acid.

It is not surprising that Ohta's acid was thought to be a single substance, for its methyl and ethyl esters consist of apparently homogeneous crystals, which have been chromatographically inseparable. It seems that methyl (and ethyl) cholate form mixed crystals with the corresponding allo esters; these mixtures were not separated even after recrystallization of their complex with ethyl 3α,7α,12α,23-tetrahydroxycholanate (Haslewood, 1961). The composition of such mixtures may be variable, but our results suggest that what have been described as esters of Ohta's acid are approximately 2:1 (w/w) methyl (or ethyl) allocholate: methyl (or ethyl) cholate. Such a composition agrees also with results of measurements of optical rotation.

Some of Ohta's (1939) and Isaka's (1940) results can be explained. Thus, Ohta was misled by elementary analyses to propose his formula $C_{27}H_{46}O_6$, and it is clear that his 'isocholic acid' was an impure sample of allocholic acid which, via the dehydro acid, gave allocholanic acid. Isaka's 'conversion' of Ohta's acid into 3α,6α-dihydroxycholanic (hyodeoxycholic) acid is inexplicable, but his 12-oxocholanic acid might have arisen from cholic acid in the original mixture.

Biological. Before its chemical nature was appreciated, Ohta's acid was first thought to be 'primitive' (Haslewood & Sjövall, 1954) and later to be possibly derived from diet (Haslewood, 1959). The latter source was excluded in the snakes in which allocholic acid was found (Haslewood, 1961), and there is no reason to doubt that allocholic acid, like cholid acid, can be formed after reduction of a cholesterol derivative in normal bile salt production. Allocholic acid occurs in a number of teleostean fish, and also in sturgeons (Haslewood, 1960) and some birds (Anderson *et al.* 1957), but it has never been found in mammals, except in a seal which

feeds on penguins (Haslewood, 1961). There is therefore a case for supposing that it represents a type of bile salt which has given way during evolution to the C-5β (cholic acid) 'modern' kind. It seems evident, however, that both C-5α and C-5β bile salts can be early forms, for scymnol and the coprostanic acids have the 5β structure and it has recently become clear that the bile alcohols ranol and cyprinol are allo compounds (Briggs & Haslewood, 1962).

It is remarkable that the existence of allo bile salts was not suspected until the present time; previous workers were perhaps handicapped by the absence of reference compounds, but also by prejudice in favour of the general occurrence of the (presumably) more lyophilic 5β substances present in the commonly available biles.

SUMMARY

1. Hydrogenation of methyl 3α,12α-diacetoxy-7-oxochol-5-enate (I) gave a mixture which, after saponification and re-esterification, yielded esters of cholic acid (about 80%) and also substances, at first believed to be methyl (or ethyl) allocholate, identical with the methyl (or ethyl) esters of Ohta's (1939) acid ('tetrahydroxynorsterocholanic acid'). The C-7β-hydroxylated esters were probably also formed.

2. Bromination, followed by saponification, of ethyl 3α-acetoxy-6-oxoallocholanate (II) gave an acid (III) identical with the supposed 3α-7β-dihydroxy-6-oxoallocholanic acid (III) originally made by hot alkaline hydrolysis of 3α,6α-dihydroxy-7-oxocholanic acid and its esters. Compound (III) was methylated and converted via the thioketal into methyl 3α-hydroxyallocholanate and other compounds.

3. Treatment with hot alkali, in aqueous dioxan, of methyl 3α,6α,12α-triacetoxy-7-oxocholanate (IV) or of ethyl 3α,12α-diacetoxy-6α-bromo-7-oxocholanate gave 3α,7β,12α-trihydroxy-6-oxoallocholanic acid (V), which, via the methyl ester thioketal, gave a mixture from which was obtained 3α,7β,12α-trihydroxyallocholanic acid (VI). Chromic oxidation of (VI) yielded 3α,7β-dihydroxy-12-oxoallocholanic acid (VII) and also 3,7,12-trioxoallocholanic acid (dehydroallocholic acid) (IX). Acid (VI) was ethylated and oxidized by *N*-bromosuccinimide and the product hydrogenated. Purification yielded the ethyl ester of (VI), but the principal product was a crystalline ester identical with a supposed isomer of ethyl allocholate isolated from snake bile (Haslewood, 1961). Saponification gave *allocholic acid.*

4. Mixtures containing methyl (or ethyl) allocholate and methyl (or ethyl) cholate (2:1, w/w) were indistinguishable from the purest available preparations of the corresponding esters of Ohta's (1939) acid, from natural sources or made as described above.

5. Allo (5α) bile acids and alcohols may represent earlier forms of bile salts which, during evolution, have been eliminated in favour of the 5β substances found in mammals.

The authors thank Professor S. Bergström for a sample of 3α,7β,12α-trihydroxycholanic acid. They are especially indebted to Professor T. Kazuno and Professor K. Yamasaki for samples of Ohta's acid and its methyl ester, and to Professor Yamasaki for unpublished information.

REFERENCES

Anderson, I. G. & Haslewood, G. A. D. (1960). *Biochem. J.* **74**, 37P.
Anderson, I. G. & Haslewood, G. A. D. (1961). *Biochem. J.* **81**, 15P.
Anderson, I. G., Haslewood, G. A. D. & Wootton, I. D. P. (1957). *Biochem. J.* **67**, 323.
Bridgwater, R. J., Briggs, T. & Haslewood, G. A. D. (1962). *Biochem. J.* **82**, 285.
Briggs, T. & Haslewood, G. A. D. (1962). *Biochem. J.* **82**, 26P.
Bush, I. E. (1952). *Biochem. J.* **50**, 370.
Fieser, L. F. & Fieser, M. (1959). *Steroids*, pp. 271–274. New York: Reinhold Publishing Corp.
Haslewood, G. A. D. (1956). *Biochem. J.* **62**, 637.
Haslewood, G. A. D. (1958). *Biochem. J.* **70**, 551.
Haslewood, G. A. D. (1959). *Ciba Found. Symp.: Biosynthesis of Terpenes and Steroids*, p. 212. London: J. and A. Churchill Ltd.
Haslewood, G. A. D. (1960). *Ann. N.Y. Acad. Sci.* **90**, 877.
Haslewood, G. A. D. (1961). *Biochem. J.* **78**, 352.
Haslewood, G. A. D. & Sjövall, J. (1954). *Biochem. J.* **57**, 126.
Haslewood, G. A. D. & Wootton, I. D. P. (1956). *Biochem. J.* **63**, 3P.
Hsia, S. L., Elliott, W. H., Matschiner, J. T., Doisy, E. A., Thayer, S. A. & Doisy, E. A. (1960). *J. biol. Chem.* **235**, 1963.
Isaka, H. (1940). *Hoppe-Seyl. Z.* **266**, 117.
Ohta, K. (1939). *Hoppe-Seyl. Z.* **259**, 53.
Takeda, K. & Igarashi, K. (1956). *J. pharm. Soc. Japan*, **76**, 867.
Takeda, K. & Igarashi, K. (1959). *J. Biochem., Tokyo*, **46**, 1313.
Takeda, K. & Komeno, T. (1954). *J. Biochem., Tokyo*, **41**, 385.
Takeda, K. & Komeno, T. (1957). *J. Biochem., Tokyo*, **44**, 249.
Takeda, K., Komeno, T. & Igarashi, K. (1954). *Pharm. Bull., Tokyo*, **2**, 352.
Yamasaki, K. (1951). *J. Biochem., Tokyo*, **38**, 93.
Ziegler, P. (1956). *Canad. J. Chem.* **34**, 1528.