SUMMARY

1. The catabolism of serum albumin labelled with ^{131}I was investigated in the isolated perfused rat liver.

2. A period of about 20 min. intervenes between the introduction of labelled rat albumin into the perfusion circuit and the appearance of non-protein-bound ^{131}I. A similar latent period is observed with heat-denatured bovine serum albumin.

3. Iodinated protein solutions were 'screened' by injection into rats for various periods before use in liver-perfusion studies, in order to remove the small proportion of molecules which are catabolized rapidly by the liver. With screening periods of up to 24 hr. the subsequent rate of catabolism shows wide variation. Labelled albumin separated either by chromatography or electrophoresis and screened for 48 or 72 hr. is broken down during 4 or 5 hr. perfusion at a constant rate which corresponds to about 14% of the total albumin breakdown *in vivo*.

4. The perfused liver is able to break down heat-denatured bovine serum albumin at 2–5 times the rate observed for screened rat albumin.

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Comparative Studies of 'Bile Salts'

11. 3α:6α:12α-TRIHYDROXYCHOLANIC ACID AND RELATED SUBSTANCES*

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There has lately been renewed interest in cholanic acids hydroxylated at C-6. Hyocholic acid from pig bile (Haslewood, 1954*a*) has been shown to be 3α:6α:7α-trihydroxycholanic acid (Haslewood, 1956; Ziegler, 1956*a*, *b*; Hsia *et al.* 1957*a*). Matschiner *et al.* (1957) have isolated from rat bile two compounds which are probably 3α:6β:7α- and 3α:6β:7β-trihydroxycholanic acids (Hsia *et al.* 1957*a*, *b*, 1958*a*; Kagan & Jacques, 1957). From the urine of surgically jaundiced rats which had been given, intragastrically, hyodeoxycholic (3α:6α-dihydroxycholanic) acid there was obtained what is probably 3α:6α:7β-trihydroxycholanic acid (Hsia *et al.* 1958*b*). Other C-6 hydroxylated 5α- and 5β-cholanic acids have been described by Gotô (1955) and Kagan (1957). Hsia *et al.* (1957*b*), Kagan (1957) and Schubert & Damker (1957) have devised methods of making substituted $\Delta^{6:7}$-cholenic acids.

The compound 3α:6α:12α-trihydroxycholanic acid is of special interest to us, first because it is one likely, on biogenetic grounds, to occur in biles and secondly because 3:6:12-trihydroxycholanic acid was stated by Ohta (1939) to have been derived by permanganate oxidation from the 'tetrahydroxy-norsterocholanic acid' first isolated by him from 'Gigi' fish bile. By direct infrared spectroscopic examination of the methyl ester of this latter compound it was concluded (Haslewood & Wootton, 1956) that it could not have the structure attributed to it by Ohta (1939), namely $C_{27}H_{46}O_6$, a 3:6:12:24-tetrahydroxy coprostanic acid; it was

* Part 10: Anderson, Haslewood & Wootton (1957).

decided nevertheless to attempt to make 3α:6α:12α-trihydroxycholanic acid for comparison.

Success in this project was first reported in a preliminary note by Takeda & Igarashi (1956). In spite of this anticipation of some of our work, we thought that it was of sufficient interest to be completed independently. The present report is of experiments, differing somewhat from those of Takeda & Igarashi (1956), which have led to the preparation of 3α:6α:12α-trihydroxycholanic acid and to confirmation of its structure. Description is also included of compounds unexpectedly obtained by another route which it was thought might lead to this substance.

As part of the work now reported, criticisms by Ziegler (1956*b*) of part of the present author's earlier work on hyocholic acid have been examined.

RESULTS

For most experiments, the starting material was ethyl 3α:12α-diacetoxy-7-oxocholanate (I). Departure from this substance was by (*a*) bromination or (*b*) preparation of its enol acetate.

(*a*) *Compounds derived after bromination of* (*I*). Bromination occurred in stages, as mentioned by Corey (1954) for the corresponding methyl ester. The final crystalline product (Hoehn & Linsk, 1945) must on Corey's (1954) findings be described as ethyl 3α:12α-diacetoxy-6α-bromo-7-oxocholanate (II). Cold alkaline hydrolysis of this in aqueous dioxan under nitrogen gave a product which could, after methylation and re-acetylation, be separated into the methyl 3α:6α:12α-triacetoxy-7-oxocholanate (III) of Takeda, Komeno & Igarashi (1954) and (in lesser yield) an isomer (IV), m.p. 196–198°, $[\alpha]_D + 89°$, not apparently encountered by these workers. A poorer yield of these products was obtained from the (non-crystalline) primary bromination product of (I).

Compound (III) with ethane-1:2-dithiol gave Takeda & Igarashi's (1956) thio ketal (m.p. about 215°) which with Raney nickel gave diacetyl methyl deoxycholate (methyl 3α:12α-diacetoxycholanate) and methyl 3α:6α:12α-triacetoxycholanate (V, R = CO·CH$_3$, R′ = Me) in respective yields of about 85 and 15 %. Ethyl 3α:6α:12α-trihydroxycholanate (V, R = H, R′ = Et) was partially acetylated and the product oxidized with chromic acid to give ethyl 3α:6α-diacetoxy-12-oxocholanate, which on Kishner–Wolff reduction gave hyodeoxycholic (3α:6α-dihydroxycholanic) acid. This work confirms Takeda & Igarashi's (1956) preparation of (V) by the thio ketal method and confirms their assignment of chemical constitution to it. No satisfactory result could be obtained from Kishner–Wolff reduction of (III) or (IV). Ethyl 3α:6α:12α-trihydroxycholanate in paper chromatography with Bush's (1952) systems ran much more slowly than ethyl cholate (3α:7α:12α-trihydroxycholanate) or ethyl hyocholate (3α:6α:7α-trihydroxycholanate).

The isomer (IV), m.p. 196–198°, with ethane-1:2-dithiol formed a thio ketal, m.p. 168–169°, $[\alpha]_D + 80°$, which with Raney nickel gave an even higher yield of diacetyl methyl deoxycholate than the thio ketal of (III) itself. After hydrolysis and re-esterification of the whole reaction product there was finally isolated a small amount of an ethyl ester which ran on paper in Bush's (1952) system B$_3$ at the same rate as ethyl 3α:6α:12α-trihydroxycholanate. Dr I. D. P. Wootton compared the infrared spectra of (III) and (IV) and reported that the spectral evidence is consistent with the suggestion that (IV) is the 6β (axial) epimer of (III).

(*b*) *Compounds from the enol acetate of* (*I*). This enol acetate can be considered (Hirschmann & Wendler, 1953) to have the $\Delta^{6:7}$ structure (VI). When it was treated with perbenzoic acid and the crude epoxide (possibly VII) hydrolysed by alkali, there was obtained in fair yield an acid, $C_{24}H_{38}O_6$ (VIII), readily giving a crystalline hydrate, m.p. about 140° and 265°, $[\alpha]_D - 10°$. Methylation and vigorous acetylation of this compound gave a methyl ester triacetate, m.p. 207–209°, $[\alpha]^D + 42°$. It appears doubtful whether either of these substances is identical with any of the products described by Takeda *et al.* (1954) as isolated after the hydrolysis of (II), although a small yield of the acid (VIII) was in one instance isolated here after such a hydrolysis.

The methyl ester triacetate of (VIII) was recovered completely after prolonged treatment with ethane-1:2-dithiol, in conditions which readily gave the thio ketals of (III) and (IV).

When acid (VIII) was treated by the Kishner–Wolff method, with anhydrous hydrazine (e.g. Moffett & Hunter, 1951), an acid $C_{24}H_{40}O_6$ (IX), m.p. about 270°, $[\alpha]_D - 9°$, was isolated together with another (X) of probable formula $C_{24}H_{38}O_5$, and m.p. > 300°. This formed an ethyl ester (apparently saturated) of m.p. 144–146°, $[\alpha]_D + 11°$, and, after methylation, was reduced by lithium aluminium hydride to (apparently) a triol, $C_{24}H_{40}O_4$, $[\alpha]_D + 10°$.

Acid (X) was also formed, in small yield, when (IX) was submitted to the above-mentioned Kishner–Wolff conditions. Chromic oxidation of (X) gave a dehydro acid, probably $C_{24}H_{34}O_5$, which was converted by Kishner–Wolff reduction into an impure crystalline mixture. The ethyl ester of (X) ran on paper chromatography in Bush's (1952) systems at about the same rate as ethyl deoxycholate (ethyl 3α:12α-dihydroxycholanate). Acids (VIII) and (IX) were resistant to the action of

periodic acid, consuming little of this reagent in the conditions used.

(c) *Nature of 'acid* A'. Ziegler (1956*b*) suggested that the 'acid *A*' derived from hyocholic and 3α-hydroxy-7-oxocholanic acids by Haslewood (1956) was impure. 'Acid *A*' has now been prepared again by hot alkaline hydrolysis of crystalline ethyl 3α:6α-diacetoxy-7-oxocholanate ($[\alpha]_D + 25°$), made in turn from diacetyl ethyl hyocholate ($[\alpha]_D + 23°$). Crystallized from methanol, 'acid *A*' finally had m.p. 226–227°, $[\alpha]_D + 35°$.

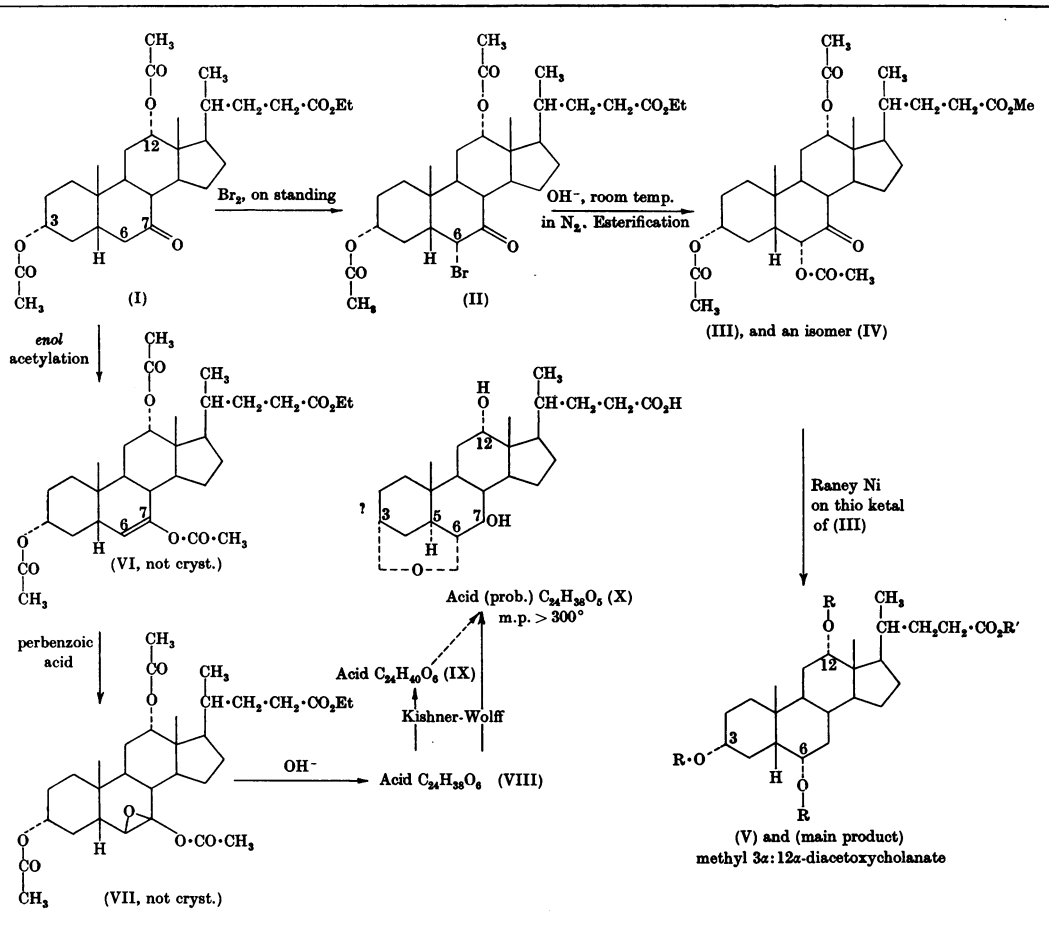
EXPERIMENTAL

General. Melting points are corrected. Optical rotations were determined in a 1 dm. micro-tube. Analyses (C, H, S, mol.wt.) were by Weiler and Strauss, Oxford. Dr I. D. P. Wootton determined infrared spectra. The Al_2O_3 was similar to that used in previous work (Haslewood, 1956), and neutralized as described by Evans & Shoppee (1953). Anhydrous hydrazine was prepared as by Barton, Ives & Thomas (1955). The 20% CrO_3 and light petroleum were as described previously (Haslewood, 1956). Raney Ni was freshly prepared, as by Adkins & Billica (1948): it was washed with water (not under H_2) until the washings gave no colour with phenolphthalein, then twice with ethanol. It was used at once. Paper chromatography with Bush's (1952) systems was as described by Haslewood (1954*b*).

Ethyl 3α:12α-*diacetoxy-7-oxocholanate* (*I*). Ethyl 3α:12α-dihydroxy-7-oxocholanate (4 g., Haslewood, 1944) in acetic acid (20 ml.) and acetic anhydride (4 ml.) was treated, with cooling to about 20°, with 8·4 N-$HClO_4$ (0·15 ml.). After 40 min., the mixture was diluted with water and twice extracted with ether. The extracts were washed several times with water, aqueous NH_3, and water and dried (Na_2SO_4). Evaporation left (I), as a colourless gum.

Compounds derived after bromination of the ester (*I*)

Bromination. Compound (I) (from 4 g. of starting ester) in acetic acid (20 ml.) was treated with 20 ml. of a solution of Br_2 (0·7 ml.) in acetic acid (30 ml.). A portion (0·05 ml.) of a solution of HBr in acetic acid was added and the mixture left at about 20°. After about 1 hr., Br_2 disappeared and HBr appeared: when this mixture was poured into water (excess), 'primary bromination product' was precipitated. This was collected, washed with water and dried *in vacuo* over $CaCl_2$. It would not crystallize. A product similarly



obtained after bromination for about 20 hr. was yellow: it readily crystallized from methanol (about 60 ml.) to give ethyl 3α:12α-diacetoxy-6α-bromo-7-oxocholanate (II), m.p. 130–132°. Yield, about 3·0–3·5 g.

Methyl 3α:6α:12α-triacetoxy-7-oxocholanate (III) and its isomer (IV). A mixture of dioxan (80 ml., A.R., or distilled over KOH), water (40 ml.) and 2N-KOH (20 ml.) was heated to about 90° with N_2 bubbling through. It was allowed to cool to about 20°, still in N_2, and to it was added the bromo compound (II) (2·8 g.). The stoppered mixture was left with occasional shaking at 20° for about 1 hr., when it became homogeneous and yellow. After about 20 hr., CO_2 (excess) was passed in and then dioxan was removed *in vacuo* at 60° or below. The aqueous residue was treated with 2N-HCl and NaCl (excess) and kept at 5° for several hours. The precipitated solid was collected, washed with water and dried *in vacuo* over $CaCl_2$. Yield, 2·2 g. This product (9·73 g.) was left with occasional mixing for 2 days in 2% (v/v) H_2SO_4 in methanol (100 ml.). The mixture was diluted with water and extracted (twice) with ether. The extract was washed with aqueous NH_3, 0·5N-NaOH and water: the washings were orange in colour and gave an oily precipitate on acidification. Evaporation of the dried (Na_2SO_4) ether left a yellow gum, which was dissolved in acetic acid (80 ml.) with acetic anhydride (16 ml.). The solution, kept at about 25° by cooling, was treated with 8·4N-$HClO_4$ (0·4 ml.). After 15 min., with occasional mixing, it was diluted with water and treated with $NaHCO_3$ (excess). The product was extracted (twice) with ether, which was washed with water and dried (Na_2SO_4). Evaporation left a brown gum (8·72 g.). This was dissolved in benzene (10 ml.) and the solution run on a column (height 21·5 cm.; diam. 35 cm.) of Al_2O_3 (160 g.) made up in benzene. Elution was as follows (fraction letter; solvent; vol.; wt. eluted): *A*: benzene, 5 l.; 4·82 g.; *B*: benzene, 5 l.; 0·20 g.; *C*: ether, 2 l.; 2·47 g.; *D*: ethanol, 3 l.; 1·20 g. Fraction *A* was treated with ether–light petroleum (1:4, v/v) and thus gave large white prisms (4·10 g.) of methyl 3α:6α:12α-triacetoxy-7-oxocholanate (III), m.p. 150–152°; $[\alpha]_D^{22}+78\pm2°$ in ethanol (*c*, 0·8). Fraction *C*, crystallized as described above, gave silky white needles (1·03 g.), m.p. 195–197°, which after recrystallization from aqueous ethanol gave white needles of (?) *methyl* 3α:6β:12α-*triacetoxy-7-oxocholanate* (IV), m.p. 196–198°; $[\alpha]_D^{22}+89\pm2°$ in ethanol (*c*, 1·0) (Found: C, 66·7; H, 8·3. $C_{31}H_{46}O_9$ requires C, 66·2; H, 8·2%). No crystalline material was isolated from fraction *D*, which was a yellow gum.

Thio ketals of compounds (III) and (IV). It was found that a considerable period was needed to form these thio ketals; if periods of 1–2 hr. or less were used, unchanged material was recovered. Conditions finally found suitable were as follows (e.g.): the ketone (III or IV, 1·2 g.) was dissolved in a mixture of ethane-1:2-dithiol (6 ml.) and BF_3 in ether (2 ml. of a '45%' commercial preparation). When the solid had all dissolved, the stoppered mixture was left with occasional mixing for at least 17 hr.; longer periods did not improve the yield of crystalline product. The mixture was then diluted with ether and the ether thoroughly washed with aqueous NH_3, 2N-NaOH and water, dried (Na_2SO_4) and evaporated. The residue crystallized at once when dissolved in benzene and treated with light petroleum (excess), giving the thio ketal. Yield, about 0·7–0·9 g. The product from (III) had m.p. 211–215°; and the above treatment of (IV) gave fluffy white needles of (?) *methyl* 3α:6β:12α-*triacetoxy-7-oxocholanate thio ketal*, m.p. 168–169°; $[\alpha]_D^{21}+80\pm1°$ in ethanol (*c*, 0·46) (Found: S, 11·5; $C_{33}H_{50}O_8S_2$ requires S, 10·0%).

Raney-nickel products from thio ketals. At least two treatments as below with fresh Raney Ni were required to obtain products not apparently containing unchanged thio ketal. A mixture of methyl 3α:6α:12α-triacetoxy-7-oxocholanate thio ketal (0·88 g., m.p. 211–215°) with fresh Raney Ni (from 10 g. of alloy) and ethanol (80 ml.) was gently boiled under reflux for 20 hr. Nickel was removed by filtration and the solvent was evaporated. The residue, which still contained some thio ketal, was again boiled under reflux in ethanol (80 ml.) with fresh Raney Ni (from 5 g. of alloy) for 17 hr. Recovery as before gave the final product as a colourless oil (0·68 g.). This was dissolved in light petroleum containing a little ether and seeded with a crystal of methyl 3α:6α:12α-triacetoxycholanate. Colourless crystals separated, and after about 2 weeks at about 20° these were collected and washed with light petroleum. Yield of methyl 3α:6α:12α-triacetoxycholanate (V, R = CO·CH_3, R′ = Me), 73 mg., m.p. 143–147°. This ester was identical with that described below.

In other experiments the final Raney Ni product (0·78 g.) was hydrolysed by boiling under reflux for 1·25 hr. with ethanol (10 ml.) and 5N-KOH (2 ml.). Ethanol was removed *in vacuo* and the residue dissolved in water. The solution was treated with 2N-HCl and NaCl (excess) and the precipitated solid collected, washed with water and dried by evaporation *in vacuo* with ethanol and benzene. Part (0·25 g.) of the residue was left at about 20° in 2% (v/v) H_2SO_4/ethanol (5 ml.) for 16 hr. The product was diluted with water and extracted (twice) with ether. The ethereal solution was washed with water, aqueous NH_3 and water, dried (Na_2SO_4) and evaporated. The crude ethyl esters (0·27 g.) were dissolved in benzene (5 ml.) and run on a column of Al_2O_3 (3 g.). Each solvent (50 ml.) eluted fractions as follows (fraction letter; solvent; wt. of eluted substance): *A*: benzene; 115 mg.; *B*: benzene; 11 mg.; *C*: ether; 100 mg.; *D*: acetone; 27 mg.; *E*: ethanol; 3 mg. Total eluted, 256 mg. Fraction *A*, with light petroleum, readily gave crystals of ethyl deoxycholate, m.p. and mixed m.p., 92–94°. On ascending paper chromatography with Bush's (1952) system B_3, fractions *D* and *E* gave spots (R_F approx. 0·05) running at the same rate as ethyl 3α:6α:12α-trihydroxycholanate, prepared by hydrolysis and re-esterification of Takeda's methyl 3α:6α:12α-triacetoxycholanate (below) by the methods described above. Run at the same time, ethyl hyocholate and ethyl cholate both had R_F approx. 0·25. Fraction *C* gave only a spot corresponding to ethyl deoxycholate: however, this fraction would not crystallize. Fraction *D* (25 mg.) in ethanol (0·5 ml.) with 5N-KOH (0·1 ml.) was warmed at about 60° for 0·5 hr. The mixture, diluted with water, was treated with 2N-HCl and NaCl (excess). The solid 3α:6α:12α-trihydroxycholanic acid (V, R = R′ = H) was collected and washed with water. As Takeda & Igarashi (1956) found, this substance (however obtained) did not crystallize. In the Hammarsten HCl test it gave a clear solution, becoming a cloudy yellow. The trihydroxycholanic acid, dried by evaporation *in vacuo* with acetone and ethanol, was left in 2% (v/v) H_2SO_4/methanol (1·5 ml.) for 16 hr. The solution was diluted with aqueous $NaHCO_3$ (excess) and the organic product recovered with ether, which was washed with water, dried (Na_2SO_4) and evaporated. The residue was

dissolved in acetic acid (0·5 ml.) with acetic anhydride (0·1 ml.) and the solution treated with 8·4N-$HClO_4$ (1 drop). After 15 min., the mixture was diluted with water and extracted with ether. The ether, washed with aqueous NH_3 and water and dried (Na_2SO_4), was removed and the residue (22 mg.) treated with light petroleum and a 'seed' of methyl 3α:6α:12α-triacetoxycholanate. Crystals (15 mg.) were formed at once; they had m.p. 145–148°, not depressed by a sample (m.p. 148–152°) of methyl 3α:6α:12α-triacetoxycholanate given by Dr K. Takeda. Both samples had the same infrared spectrum in the region 900–1200 cm.$^{-1}$. Neither sample depressed the m.p. of methyl 3α:6α:12α-triacetoxy-7-oxocholanate (III, m.p. 150–152°), but this last substance gave a different infrared spectrum.

When the thio ketal (0·19 g., m.p. 168–169°) of (IV) was treated as above (twice) with Raney Ni in ethanol, the final product was a colourless oil (0·15 g.). This crystallized from a little light petroleum, giving diacetyl methyl deoxycholate (Koechlin & Reichstein, 1942) as almost the sole product. A sample was separated by chromatography on Al_2O_3, as the ethyl esters of the corresponding acids (prepared by hydrolysis and re-esterification of the Raney Ni product as described above). The ethyl esters (125 mg.) in benzene (1·0 ml.) were put on a column of Al_2O_3 (1·3 g.). Benzene (50 ml.) eluted 76 mg., ether (50 ml.) 46 mg., and acetone (50 ml.) 2·5 mg.

The residues left after evaporation of solvents from all eluates were run in Bush's (1952) system B_3 on paper. The ether residue gave a very faint, and the acetone residue a strong, spot corresponding to ethyl 3α:6α:12α-trihydroxycholanate. The infrared spectra of the acetone residue and of ethyl 3α:6α:12α-trihydroxycholanate were compared and found to be almost identical.

Ethyl 3α:6α-diacetoxy-12-oxocholanate and its conversion into hyodeoxycholic acid. Methyl 3α:6α:12α-triacetoxycholanate was hydrolysed and the acid re-esterified with ethanol as described above. The resulting (non-crystalline) ethyl 3α:6α:12α-trihydroxycholanate (26 mg., V, R = H, R′ = C_2H_5) was left for 16 hr. at about 18° in pyridine (0·4 ml.) with acetic anhydride (0·4 ml.). The mixture was diluted with 2N-HCl and extracted twice with ether. The ether was washed with water, dried (Na_2SO_4) and evaporated. The residue in acetic acid (0·5 ml.) was treated with 20% CrO_3 (0·1 ml.). After 10 min. water and NaCl (excess) were added and the product extracted (twice) with ether. The ether, washed with water, aqueous NH_3 and water, and dried (Na_2SO_4) was evaporated. The residue with light petroleum at once formed crystals, which, after recrystallization from benzene–light petroleum, formed fine white needles of *ethyl 3α:6α-diacetoxy-12-oxocholanate*, m.p. 177–179° (Found: C, 70·0; H, 9·2. $C_{30}H_{46}O_7$ requires C, 69·5; H, 8·9%). This substance (15 mg.) was added to a small metal bomb containing a solution of Na (30 mg.) in ethanol (1 ml.) with hydrazine hydrate (0·1 ml. of '99/100%'). The bomb was sealed and heated at 190–195° for 17 hr. The contents were diluted with water and treated with 2N-HCl and NaCl (excess). The solid product was collected and crystallized twice from ethyl acetate. It then gave glistening white needles, m.p. 197–198°, not depressed by authentic hyodeoxycholic acid (m.p. 198–199°). A sample of each acid was methylated with diazomethane and their infrared spectra were compared and found to be identical in the region 900–1200 cm.$^{-1}$.

Compounds derived from the enol acetate of (I)

Ethyl $\Delta^{6:7}$-3α:7:12α-*triacetoxycholenate (VI).* Ethyl 3α:12α-dihydroxy-7-oxocholanate (4 g.) was acetylated by the $HClO_4$ method as described above. The product (I) was dried *in vacuo* over H_2SO_4. It was then dissolved in dry benzene (80 ml.) with crystalline toluene-*p*-sulphonic acid (0·6 g.) and *iso*propenyl acetate (8 ml.). During 5 hr. the mixture was slowly distilled, a fresh portion (4 ml.) of *iso*propenyl acetate being added after 3 hr. In all, about 45 ml. of distillate was collected. Most of the remaining solvent was removed *in vacuo* and the residue dissolved in ether. This was well washed with water, aqueous $NaHCO_3$ and water, and dried (Na_2SO_4) and evaporated. The residue (probably largely VI) was a light-brown gum.

Perbenzoic acid product from (VI). A freshly prepared solution of perbenzoic acid (3·77 g., approx. 4·5 mol.) in $CHCl_3$ (60 ml.) was added to the preparation of (VI) described above. When all had dissolved, the mixture was left at about 25° for 18 hr. Titration showed that in such conditions about 10% more than the theoretical amount of perbenzoic acid needed to form the epoxide (e.g. VII) had disappeared. The reaction mixture was diluted with water and treated in succession with KI, 2N-H_2SO_4 and aqueous $Na_2S_2O_3$ (excess); it was then extracted twice with ether. The ether was washed with water, aqueous NH_3 and water, dried (Na_2SO_4) and evaporated, leaving the epoxide (probably mainly VII) as an almost colourless gum.

Acid $C_{24}H_{38}O_6$ *(VIII) and derivatives.* (i) Hot alkaline hydrolysis of (VII). The product (VII) (from 4 g. of ethyl 3α:12α-dihydroxy-7-oxocholanate) was dissolved in a mixture of ethanol (40 ml.) and 5N-KOH (20 ml.). The solution was boiled under reflux for 1 hr., after which water (30 ml.) and 5N-KOH (10 ml.) were added. After a further 1·5 hr. boiling under reflux, ethanol (30 ml.) was allowed to distil for 0·5 hr. The cooled mixture was then filtered and treated with 5N-HCl and NaCl (excess). After standing overnight at about 5°, the precipitated solid was collected, washed with water and dissolved in ethanol (vol. 150 ml., after the solution had been again filtered). To the hot solution was gradually added hot water (600 ml.). Crystals formed at once. After 20 hr., finally at 5°, the crystals were collected and dried *in vacuo* over $CaCl_2$. Yield 2·74 g. This substance was a hydrate: it melted at about 140°, recrystallized at about 155° and finally decomposed at about 260°. It did not crystallize from organic solvents, but could be purified as the crystalline hydrate from aqueous ethanol. The *hydrate of acid (VIII)* had $[\alpha]_D^{22} - 10 \pm 2°$ in ethanol (*c*, 1·7) [Found (on a sample dried at 80° *in vacuo*): C, 66·0; H, 9·3%; equiv.wt. (titration) 467. $C_{24}H_{38}O_6,H_2O$ requires C, 65·5; H, 9·1%; equiv.wt. (one CO_2H) 440]. This acid (422 mg.) in methanol (7 ml.) was left with aqueous 0·548M-HIO_4 (2 ml., 1·1 mol.) at about 22° for 22 hr. After the usual treatment, it was found that HIO_4 equiv. to about 0·025 atom of oxygen had been used up. From the reaction mixture, starting material (0·227 g.) was recovered as the crystalline hydrate.

(ii) Cold alkaline hydrolysis of (VII). The product (VII) (from 4 g. of starting ester) was dissolved in ethanol (40 ml.) and treated at about 20° with 2N-KOH (40 ml.). The orange solution was left with occasional shaking for 16 hr.; it was then diluted with water and filtered. The filtrate was treated with 5N-HCl and NaCl (excess) and left at 5° for 24 hr. The solid precipitate was collected,

washed with water and dried over $CaCl_2$. The product (yield, 3·54 g., $[\alpha]_D^{20}$ approx. +19° in ethanol, *c*, 1·2) was left for 70 hr. in 2% (v/v) H_2SO_4 in methanol (50 ml.). The solution was diluted with water and extracted (twice) with ether. The ether was washed with water, aqueous NH_3 and water, dried (Na_2SO_4) and evaporated. The residue in acetic acid (20 ml.) with acetic anhydride (4 ml.) was treated, with cooling to about 20°, with 8·4N-$HClO_4$ (0·1 ml.). After 10 min. the mixture was diluted with water and extracted with ether. The extract, purified and dried as above, gave, on evaporation and treatment of the residue with cold ether, white needles (1·7 g., m.p. 201–203°) of the methyl ester triacetate of (VIII), described below. The methyl ester of (VIII) was formed from the hydrate of acid (VIII) by methylation with diazomethane or with H_2SO_4–methanol as described above; this ester could be acetylated by the $HClO_4$ method, as above, or, with difficulty, by prolonged (20 hr.) heating at 95° with equal volumes of pyridine and acetic anhydride. In both cases, the final product crystallized from aqueous ethanol or light petroleum–benzene as long white needles of the *triacetyl methyl ester* of acid (VIII), m.p. 207–209°, $[\alpha]_D^{21}$ +42±2° in chloroform (*c*, 1·7) (Found: C, 66·6; H, 8·4. $C_{31}H_{46}O_9$ requires C, 66·2; H, 8·2%). This substance was recovered quantitatively after prolonged (24 hr.) treatment with ethane-1:2-dithiol and ethereal BF_3, as described above. Hydrolysis with boiling ethanolic KOH reconverted it into the acid (VIII).

Kishner–Wolff products from the acid (VIII). The following method gave both (IX) and (X). The hydrate of (VIII) (0·3 g.) was dehydrated by evaporation *in vacuo* with ethanol–benzene. The residue, in ethanol, was transferred to a small metal bomb and solvent evaporated. To the bomb was added dry ethanol (7 ml.) and Na (0·3 g.). When the Na had dissolved, anhydrous hydrazine (0·8 ml.) was added and the bomb was sealed and heated at 180±1° for 19 hr. The contents were then washed out with water, and the solution treated with 2N-HCl and NaCl (excess). The white crystalline solid was collected and washed with water. It was dissolved in ethanol (15 ml.) and the hot solution diluted with water (30 ml.). On standing, acid (X) was precipitated as white needles (35 mg.); these were collected and washed with water–ethanol (2:1, v/v). The liquors were evaporated *in vacuo* and the residue crystallized from acetone–ethyl acetate to give acid (IX), yield 130 mg.

A higher yield of acid (X) was given by the method of Moffett & Hunter (1951). The method of Barton *et al.* (1955) gave only acid (IX).

Acid (IX). This substance crystallized from acetone–ethyl acetate as large colourless prisms, decomp. about 270°; Hammarsten HCl test, clear yellow; $[\alpha]_D^{22}$ −10±1° in ethanol (*c*, 1·5) (Found: C, 67·9; H, 9·2. $C_{24}H_{40}O_6$ requires C, 67·9; H, 9·4%). This acid (0·1 g.) in methanol (7 ml.) was left with aqueous 0·548M-HIO_4 (1 ml.) for 19 hr. at about 25°. Titration showed that almost no HIO_4 had been consumed. Oxidation of acid (IX) in acetic acid at room temperature with 20% CrO_3 (excess) gave a product which could be extracted by ether after dilution of the mixture with water. Evaporation of the washed ether gave a crystalline product of m.p. 244–248° (decomp.); it was not further purified. A similar product (m.p. 225–241°) was similarly isolated from acid (VIII). Both products gave only faint yellow colours on treatment with aqueous NaOH: this finding probably indicates that they do not contain keto groups at C-6 in a cholane nucleus (Haslewood, 1956). When acid (IX) was submitted to Kishner–Wolff conditions (method of Moffett & Hunter, 1951) there was obtained a small yield of acid (X), isolated as described above.

Acid (X). This acid was recrystallized from ethyl acetate. It formed short white needles which decomposed, with sublimation, at about 295–310°. Analysis was unsatisfactory, probably because of these properties (Found: C, 69·6; H, 9·2. $C_{24}H_{38}O_5$ requires C, 70·9; H, 9·4%). The acid was sparingly soluble in all neutral solvents; 40 mg. of it in a mixture of ethanol (4 ml.) and H_2SO_4 (0·1 ml.), was warmed to about 60°, until it dissolved. After some days, the solution was diluted with aqueous $NaHCO_3$ (excess). The solid product was collected, washed with water and crystallized from aqueous ethanol, giving the *ethyl ester* of acid (X) as colourless, glistening leaflets, m.p. 144–146°. $[\alpha]_D^{23}$ +11±2° in ethanol (*c*, 1·0) [Found: C, 71·7; H, 9·6%; mol.wt. (Rast) 518. $C_{26}H_{42}O_5$ requires C, 71·9; H, 9·7%; mol.wt. 434]. This ester gave no colour with tetranitromethane in $CHCl_3$. On paper chromatography with Bush's (1952) system A, it ran at about the same rate as ethyl deoxycholate. On alkaline hydrolysis it was reconverted into the original acid (X). The corresponding methyl ester, partially purified, had m.p. 178–184°: acetylation of this by the $HClO_4$ method finally gave a gelatinous solid which was washed with water and then dried at 95° *in vacuo* for analysis [Found: C, 68·6; H, 8·8; $C_{29}H_{44}O_7$ (diacetate) requires C, 69·0; H, 8·7%]. $LiAlH_4$ product: acid (X) (0·1 g.), was suspended in methanol and methylated with diazomethane (excess). The solvent was removed *in vacuo* and the residue treated with dry ether (10 ml.) and powdered $LiAlH_4$, added in two portions (each 0·25 g.). The mixture was gently boiled under reflux for 1·25 hr. and then cooled in ice and decomposed with ice and 2N-HCl. The product was extracted (twice) with ether and the ether washed with water, aqueous NH_3 and water and dried (Na_2SO_4). Evaporation left a residue which crystallized at once with ether, giving small white needles (48 mg.) of (probably) the (hydrated) C-24 *alcohol* corresponding to (X), m.p. 141–142°, partly recrystallizing and remelting at 181–184°. $[\alpha]_D^{22}$ +10±2° in ethanol (*c*, 1·0) [Found C, 70·4; H, 11·0%; mol.wt. (Rast) 383. $C_{24}H_{40}O_4,H_2O$ requires C, 70·25; H, 10·2%; mol.wt. 410].

Dehydro acid (X). Acid (X) (0·1 g.) was suspended in acetic acid (1 ml.) and 20% CrO_3 (0·3 ml.) added. Acetic acid (1 ml.) was added after 5 min. and the mixture gently agitated until all had dissolved (30 min.). After a further 30 min. it was treated with water and NaCl (excess). The crystalline precipitate was collected, washed with water and recrystallized from aqueous ethanol, giving a product m.p. 243–246° (decomp.) [Found: C, 71·4; H, 8·9%; mol.wt. (Rast) 346. $C_{24}H_{34}O_5$ requires C, 71·6; H, 8·5%; mol.wt. 402]. When this substance was subjected to Kishner–Wolff conditions, the product was a crystalline acid, the m.p. of which could be raised by recrystallization to 279–285° [Found: C, 72·7; H, 9·4. $C_{24}H_{38}O_3$ requires C, 77·0; H, 10·2%]. Clearly this (impure) substance cannot be mainly the 'stem' acid, $C_{24}H_{38}O_3$, derived by reduction to :CH_2 of two :CO groups in the dehydro-acid $C_{24}H_{34}O_5$ (above): reduction of one such group to :CH_2 and of another to :CH·OH would give $C_{24}H_{38}O_4$ (requires C, 73·8; H, 9·7%).

'Acid A' from hyocholic acid

Crystalline diacetyl ethyl hyocholate (prepared as described by Haslewood, 1956) had $[\alpha]_D^{20}+23\pm2°$ in ethanol (*c*, 0·9). Oxidation with CrO_3 as previously described gave ethyl 3α:6α-diacetoxy-7-oxocholanate, which had $[\alpha]_D^{21}+25\pm2°$ in ethanol (*c*, 0·8). This confirms the previously made statement that oxidation to keto of :CH·OH at C-7 in some hyocholic acid derivatives affects the $[M]_D$ very slightly: the original evidence for this statement was based partly on observations now known to be faulty. A solution of ethyl 3α:6α-diacetoxy-7-oxocholanate (0·5 g., m.p. 117–120°) in ethanol (10 ml.) and 5N-KOH (1·5 ml.) was boiled under reflux for 2 hr. The cooled (yellow) mixture was treated with water, 2N-HCl and NaCl (excess). It was kept overnight at 5° and the solid precipitate was finally collected, washed with water and crystallized from aqueous ethanol. Yield of white needles, 0·21 g., m.p. about 222° (decomp.). Two recrystallizations from methanol gave white needles of constant m.p., 226–227° (decomp.), $[\alpha]_D^{24}+35\pm1°$ in ethanol (*c*, 1·2) [Found (titration): equiv.wt. 394. $C_{24}H_{38}O_4$ (one CO_2H) requires equiv.wt. 406]. Hence the original preparation (Haslewood, 1956), of 'acid *A*', which had m.p. 211–215°, $[\alpha]_D^{23}+12°$, was impure. The properties of 3α:6α-dihydroxy-7-oxocholanic acid as given by Takeda *et al.* (1954), Ziegler (1956*b*) and Hsia *et al.* (1957*a*) are quite different.

DISCUSSION

(*a*) 3α:6α:12α-*Trihydroxycholanic acid and derivatives.* Derivatives of this compound gave infrared spectra quite different from those of cholic and 'tetrahydroxynorsterocholanic acid'. This finding strengthens further the evidence against the structure proposed by Ohta (1939) and Isaka (1940) for this last acid. Moreover, none of the substances now described showed a positive (blue or purple) Hammarsten (HCl) reaction; this is given strongly by 'tetrahydroxynorsterocholanic acid' and was reported by Ohta (1939) to be characteristic also of his '3:6:12-trihydroxycholanic acid'.

It is obviously possible that the isomer (IV) of (III) is methyl 3α:6β:12α-triacetoxy-7-oxocholanate. This view is consistent with the optical-rotation findings, for the rotations of (IV) and its thio ketal are more positive than those of (III) and its thio ketal (Takeda & Igarashi, 1956, and present work). Since the contribution of a 6β-hydroxyl group is known to be much more positive than that of a 6α-OH group in the cholane series (Fieser & Fieser, 1949), these observations support the idea that (IV) may be the 6β-OH epimer of (III); however, 'vicinal' effects in the present case are uncertain, and further chemical evidence is desirable.

(*b*) *Products derived from the enol acetate (VI).* It seems probable that the perbenzoic acid product from (VI) is in fact the β-oxide (VII), for the α-side of the molecule in (VI) offers considerable hindrance to the approach of the reagent. (Compare, however, Henbest & Wilson, 1957; Albrecht & Tamm, 1957). Hot or cold hydrolysis of (VII) gave the same products; i.e. derivatives of the acid $C_{24}H_{38}O_6$ (VIII), and hence this can hardly be of the type of 'acid *A*' (discussed below) derived only by hot alkaline treatment. One Kishner–Wolff product (IX, $C_{24}H_{40}O_6$) from (VIII) is almost certainly simply the corresponding secondary alcohol (at C-7); indeed (VIII) and (IX) on oxidation with cold chromic acid were converted into (apparently) the same (impure) reaction product.

Acid (X), $C_{24}H_{38}O_5$ appears to be a product of strongly dehydrating conditions on (IX); it seems to be monomeric. It apparently still contains a secondary hydroxyl group in the hindered C-7 position, for Kishner–Wolff treatment of its chromic acid oxidation product (dehydro acid, $C_{24}H_{34}O_5$) failed to give the expected 'stem' acid $C_{24}H_{38}O_3$, by conversion of two :CO groups into :CH_2.

Formula (X) appears at least possible, for molecular models suggest that a fairly close approach of 3α- and 6α-hydroxyl groups can readily take place in the 5α series. No formula has been devised which will postulate retention of the 5β (cholane) structure in (VIII) and later products, and explain their properties.

It must be emphasized that formula (X) is largely speculative. It depends on the assumption of (VI) for the enol acetate of (I), and postulates, in (VII) → (VIII), an inversion at C-5. It implies that (VIII) and (IX) are, respectively, 3α:6:12α-trihydroxy-7-oxo- and 3α:6:7:12α-tetrahydroxy-*allo*(5α)-cholanic acids. If this is so, it is curious that they did not react with periodic acid (see, however, Hsia *et al.* 1957*b*). Formula (X) cannot be regarded as providing more than a working hypothesis for future studies.

The keto group in acid (VIII) is more hindered than any in steroids known to the present writer.

(*c*) '*Acid* A'. 3α:6α-Dihydroxy-7-oxocholanic acid has now been prepared in three Laboratories (Takeda *et al.* 1954; Ziegler, 1956*b*; Hsia *et al.* 1957*a*). It is converted by hot alkali into a mixture from which pure 'acid *A*' can be isolated. For this substance, Ziegler (1956*b*), following Takeda *et al.* (1954), suggested the formula 3α:7β-dihydroxy-6-oxo-*allo*cholanic acid. Chemical evidence for this formula is incomplete. Later considerations of the structure of this compound do not affect significantly the evidence indicating the structure of hyocholic acid as 3α:6α:7α-trihydroxycholanic acid.

SUMMARY

1. Careful alkaline hydrolysis of ethyl 3α:7α-diacetoxy-6α-bromo-7-oxocholanate has given, after appropriate further treatment, methyl

3α:6α:12α-triacetoxy-7-oxocholanate and an isomer, probably methyl 3α:6β:12α-triacetoxy-7-oxocholanate. The thio ketal of the 6α-compound with Raney nickel gave derivatives of deoxycholic acid, and, in minor yield, of 3α:6α:12α-trihydroxycholanic acid. This last acid has been converted into hyodeoxycholic acid. These results confirm and extend those of Takeda & Igarashi (1956). There is no doubt that 3α:6α:12α-trihydroxycholanic acid is not the substance ('3:6:12-trihydroxycholanic acid') obtained by Ohta (1939) from 'tetrahydroxynorsterocholanic acid'.

2. The enol acetate of ethyl 3α:12α-diacetoxy-7-oxocholanate has been prepared and treated with perbenzoic acid. Hydrolysis of the epoxide gave, in fair yield, an acid $C_{24}H_{38}O_6$, which in Kishner–Wolff condition was converted into two other acids, $C_{24}H_{40}O_6$ and (probably) $C_{24}H_{38}O_5$. The chemical reactions of these substances have been explored, and it is suggested that a possible formula for the acid $C_{24}H_{38}O_5$ is (X).

3. Crystalline diacetyl ethyl hyocholate, $[\alpha]_D$ + 23°, has been converted into ethyl 3α:6α-diacetoxy-7-oxocholanate, $[\alpha]_D$ + 25°. Hot alkaline hydrolysis of this gave, after purification, a new preparation of 'acid *A*' (Haslewood, 1956), m.p. 226–227°, $[\alpha]_D$ + 35°. Hence, as Ziegler (1956*b*) suggested, the original 'acid *A*' was impure. It could not have been chiefly 3α:6α-dihydroxy-7-oxocholanic acid.

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