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Comparative Studies of 'Bile Salts'

9. THE ISOLATION AND CHEMISTRY OF HYOCHOLIC ACID*

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The bile of the domestic pig is known to contain 3α:6α-dihydroxycholanic acid (hyodeoxycholic acid; Windaus & Bohne, 1923), 3α:7α-dihydroxycholanic acid (chenodeoxycholic acid; Ido & Sakurai, 1939), 3β:6α-dihydroxycholanic acid (Kimura, 1937) and 3α-hydroxy-6-oxo*allo*cholanic acid (or 3α-hydroxy-6-oxocholanic acid; Fernholz, 1935), all probably in conjugated form. Shimizu (1953) isolated 3α-hydroxycholanic (lithocholic) acid from the bile, and Trickey (1950) suggested that an acid of formula $C_{27}H_{44}O_6$ might be present. Pig bile acids are largely conjugated with glycine, but taurine derivatives also occur (Haslewood & Sjövall, 1954). The glycine conjugates were examined chromatographically by Haslewood & Sjövall (1954), who detected a substance which on paper ran rather more slowly than glycocholic acid. The parent trihydroxy acid, $C_{24}H_{40}O_5$, probably corresponding to this compound, was later isolated (Haslewood, 1954*a*) and named hyocholic acid. The present report describes the isolation and properties of this substance, and gives an account of experiments which have almost entirely elucidated its chemical constitution. In this work unexpected difficulties were encountered.

RESULTS

Pig bile salts were hydrolysed by recognized methods in an autoclave or, by the use of ethylene glycol, at atmospheric pressure. The ethylated crude bile acids were separated on alumina columns, and fractions were examined by paper chromatography as previously described (Haslewood, 1954*b*).

* Part 8: Haslewood & Sjövall (1954).

Esters of hyodeoxycholic and chenodeoxycholic acids were easily recognized. There were also spots corresponding to at least two unidentified substances. One of these remains unidentified, but is not ethyl 3β:6α-dihydroxycholanate or ethyl 3α-hydroxy-6-oxo*allo*cholanate; the other was shown to be ethyl hyocholate. A simple chromatographic process then permitted the isolation in substance of hyocholic acid, which could also be obtained, without chromatography, from crude 'hyodeoxycholic acid', as usually prepared. Girard separation of the ethylated bile acids removed ethyl 3α-hydroxy-6-oxo*allo*cholanate almost quantitatively in a single operation; it did not remove ethyl hyocholate. Hyocholic acid was converted into a crystalline ethyl ester, diacetyl ethyl ester, triacetyl methyl ester, triacetyl ethyl ester and other derivatives, discussed below. Triacetyl methyl hyocholate and triacetyl ethyl hyocholate readily formed characteristic crystals, resembling those of cholesterol. The triacetyl ethyl ester was useful in the isolation of hyocholic acid from mixtures.

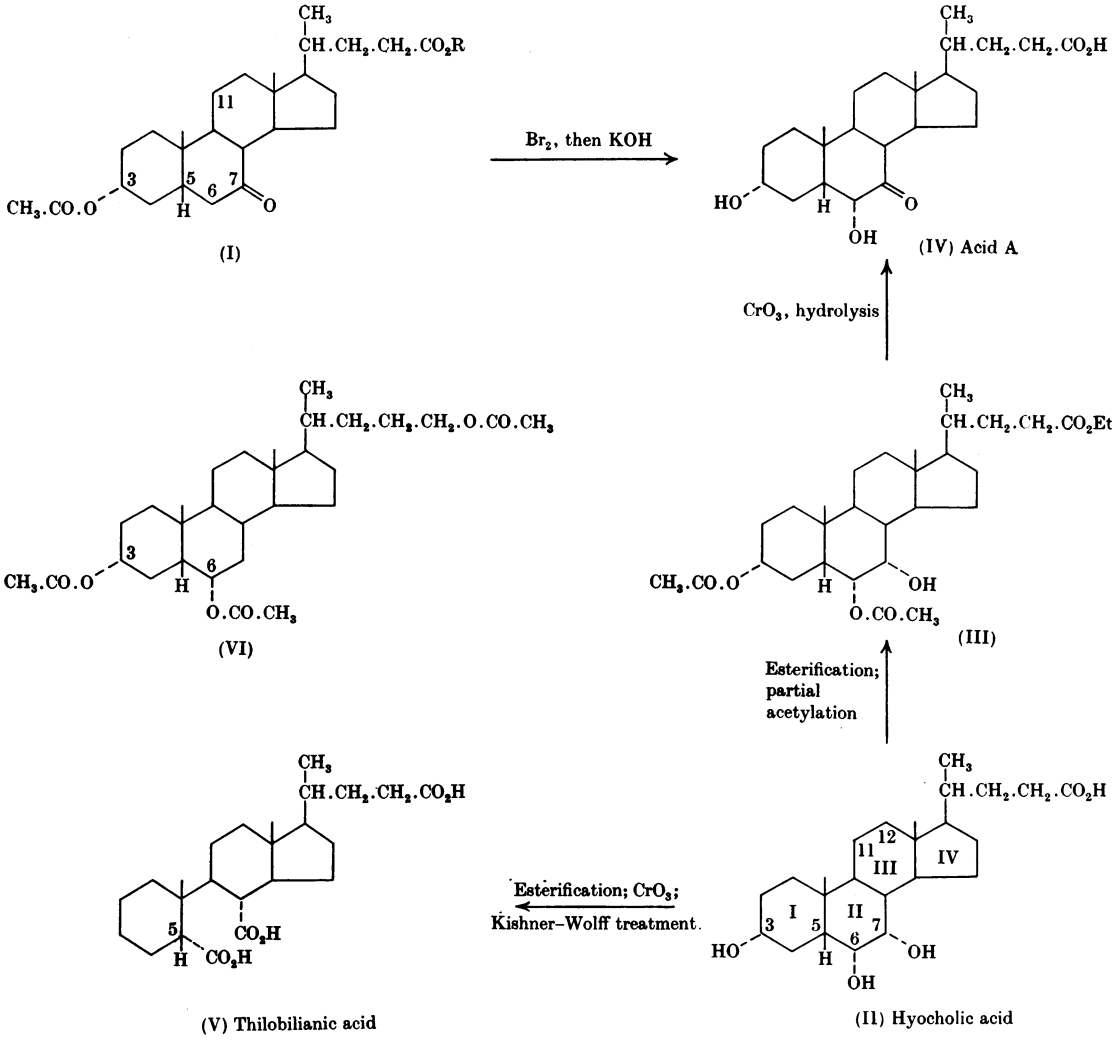
The most obvious property of hyocholic acid itself was its similarity to hyodeoxycholic acid, from which it could not easily be separated by ordinary crystallization. Purified hyocholic acid had m.p. 188–189° and $[\alpha]_D + 5{\cdot}5°$ in ethanol; the mixed m.p. with hyodeoxycholic acid (m.p. 197–198°, $[\alpha]_D + 7°$) was 184–196°. Other mixtures of hyodeoxycholic and hyocholic acids melted sharply; for example, a laboratory specimen of 'hyodeoxycholic acid' of m.p. 191–192° was shown to contain at least 20% of hyocholic acid. Much work may have been done in the past on 'hyodeoxycholic acid' contaminated in this way.

Hyocholic acid on chromic oxidation gave a (non-crystalline) product which with alkali gave a yellow colour. Windaus (1926) observed this behaviour with dehydrohyodeoxycholic (3:6-dioxocholanic) acid, and Hoehn, Linsk & Moffett (1946) similarly reported that 6-oxocholanic acid dissolved in aqueous sodium hydroxide with development of a yellow colour. This colour is presumably associated with enolization of the C-6 oxocholanic acids, and its appearance in the present case was regarded as evidence for the presence in hyocholic acid of a hydroxyl group at C-6 in a cholane nucleus.

Almost all known indubitably 'native' bile acids are 3α-hydroxy compounds, and hyocholic acid was thus provisionally formulated as a 3α:6:x-trihydroxycholanic acid.

Mild acetylation of ethyl hyocholate gave a diacetate; complete acetylation could be effected by the perchloric acid method or after prolonged heating. The diacetate was converted by chromic oxidation into a different substance of almost identical elementary composition; this substance was presumably a ketone (or aldehyde), but it did not form a Girard complex or a semicarbazone, and gave no recognizable product on Clemmensen or Kishner–Wolff reduction. Hence the third hydroxyl group in hyocholic acid was (*a*) primary or secondary, (*b*) difficult to acetylate, and (*c*) convertible into a very unreactive keto (or aldehyde) group.

An 11α-hydroxyl group would have properties (*a*) and (*c*), and the possibility of the existence of such a group was supported by optical-rotation measurements. The (presumed) 3α:6-dihydroxy-x-oxocholanic acid (A) derived from the unreactive



'ketone' decomposed at about 215° and had an $[M]_D$ (about +50°), which differed little from that of hyocholic acid (about +22°). These findings excluded position C-12 and supported C-11 for a hydroxyl group having the properties enumerated (Barton & Klyne, 1948; Haslewood & Wootton, 1951). No other position, as far as was then known, could be seriously considered and hyocholic acid was tentatively formulated as 3α:6α:11α-trihydroxycholanic acid (Haslewood, 1954*a*, 1955).

However, as evidence was secured, this possibility seemed more and more unlikely. The diacetate did not form a *p*-tosylate, and could not be satisfactorily dehydrated with $POCl_3$; the derived 'ketone' on catalytic hydrogenation was converted not into the expected 11β-hydroxy compound but largely into the original diacetate. Reduction of triacetyl ethyl hyocholate with $LiAlH_4$ gave, in good yield, a tetraol, presumably 3α:6:x:24-tetrahydroxycholane, which gave a triacetate and a tetraacetate. The triacetate, like diacetyl ethyl hyocholate, could be oxidized to a ketone (or aldehyde) unreactive towards semicarbazide and giving no recognizable product after treatment by the Kishner–Wolff method. It could not thus be converted into 3α:6α:24-triacetoxycholane (VI) prepared after $LiAlH_4$ reduction of ethyl hyodeoxycholate. In other respects, also, the $LiAlH_4$ product (tetraol) from triacetyl ethyl cholate behaved like the parent ester, in that its triacetate could not be satisfactorily dehydrated with $POCl_3$ and the derived 'ketone' regenerated the original triacetate almost quantitatively on reduction with $LiAlH_4$.

Finally, it was found that cold chromic acid oxidation of ethyl hyocholate gave, in substantial yield, an *acidic* product, which was subsequently converted by Kishner–Wolff reduction and hydrolysis into a substance having the properties of thilobilianic acid (V). This observation could by no means be reconciled with the 3α:6α:11α-trihydroxy formula, but indicated that two of the hydroxyl groups must occupy adjacent positions in ring II.

Among the new possibilities for a hyocholic acid formula was 3α:6α:7-trihydroxycholanic acid (II). When ethyl 3α-acetoxy-7-oxocholanate (I, R = Et) or the acid (I, R = H) was brominated and the product boiled with methanolic potassium hydroxide, there was isolated an acid, decomposing at about 215°, which was identical with the acid A mentioned above. This acid is probably 3α:6α-dihydroxy-7-oxocholanic acid (IV, see Discussion), and hyocholic acid is therefore 3α:6α:7-trihydroxycholanic acid (IV). Some evidence was obtained that hyocholic acid formed an acetonide, although no crystalline derivative could be made; acetonide formation would indicate that the hydroxyl groups at C-6 and C-7 are probably in the *cis* configuration, i.e. the hydroxyl group at C-7 is in the α-position.

EXPERIMENTAL

General. Melting points were determined on a Kofler-block type of apparatus and are corrected. Al_2O_3 was Spence 'Type H' (Peter Spence, Widnes) except where otherwise stated. Light petroleum had b.p. 40–60°. 20% CrO_3 was a solution of about 20 g. of CrO_3 in a minimal vol. of water made, with mixing, to 100 ml. with acetic acid. Microanalyses (C, H) were by Drs Weiler and Strauss, Oxford.

Esterification. Process *A* consisted in leaving the dried acid (1 part) in methanol or ethanol (10 parts) containing 2% (v/v) H_2SO_4 for at least 16 hr., with occasional shaking, sufficient to cause complete solution and mixing. The mixture was then diluted with water and extracted 2–3 times with ether. The ether was washed with water, aqueous ammonia and water, dried (Na_2SO_4) and evaporated; the residue was 'esters'. Acidification of the aqueous ammonia washings gave 'unesterified acids'.

Hydrolysis of pig bile salts

(*a*) *At ordinary pressure.* Bile salts (50 g., prepared as described by Haslewood & Wootton, 1950) were dissolved in a mixture of KOH (25 g.), water (120 ml.) and ethylene glycol (250 ml.). The solution was boiled for 3 hr., with loss of vapour, until the temp. rose to 140°. A reflux condenser was then fitted and the mixture simmered, gently to avoid frothing, for a further 10 hr. at 138–142°. The cooled product was diluted with water (1·5 l.) and acidified with 5N-HCl (excess). After 3 days, the liquors were decanted, and the washed residue, dried by evaporation with ethanol, was stirred with ethyl acetate. The filtered solution was evaporated and the residue dissolved in 50% (v/v) acetone–ethyl acetate. Crystals slowly formed, and after about 20 days at about 5° these were collected and washed with cold 50% (v/v) acetone–ethyl acetate. Yield, about 5 g., m.p. 185–188°. This material was later shown to be approximately 80% (w/w) hyodeoxycholic–hyocholic acids. The ethyl acetate liquors were evaporated and the residue (33 g.) ethylated by process *A*, giving 'ethyl esters' (32 g.) as a dark-brown gum.

(*b*) *At increased pressure.* Bile salts (40 g.) were dissolved in a 500 ml. beaker with KOH (24 g.), water (120 ml.) and ethanol (120 ml.). The beaker, covered with a clock glass, was placed in a 'pressure cooker' containing about 800 ml. of water, and the cooker was sealed and heated at 35–40 lb. pressure/in.^{2} for 6 hr. When the cooled cooker was opened, it was found that part of the alkaline 'bile acid' solution had frothed out of the beaker. The frothed liquors were collected separately; acidification of these gave a solid precipitate (2·1 g.) which, after washing and drying, separated easily from ethyl acetate as crystals (0·5 g.) of m.p. 182–185°, subsequently shown to be approximately 80% (w/w) hyodeoxycholic–hyocholic acids. Acidification of the diluted contents of the beaker gave the 'bile acids'; these were collected, washed, evaporated to dryness with ethanol *in vacuo* and esterified by process *A*. Yield, 28 g. of 'ethyl esters'.

Separation of 'ethyl' esters and bile acids

Preliminary separation. The 'esters' (8·7 g., prepared as under (*a*) above) were dissolved in benzene (about 20 ml.) and the solution was poured on a column (diam. 2·9 cm.) of

Al_2O_3 (100 g.) prepared in benzene. Fractions were eluted and each fraction was examined by paper chromatography with Bush's (1952) system *A* as previously described (Haslewood, 1954*b*). Results are shown in Table 1. Unknown X ran at rather more than half the rate of ethyl chenodeoxycholate and unknown Z rather more slowly than ethyl cholate. X has not yet been identified; it cannot be ethyl 3β:6α-dihydroxycholanate or ethyl 3α-hydroxy-6-oxo*allo*cholanate, for these were not detected on chromatograms under the conditions described above. Z corresponded in position to ethyl hyocholate. In some chromatograms there was an indication of an unknown Y, running at a rate between those of ethyl hyodeoxycholate and ethyl hyocholate.

The crystals in fraction II were isolated, recrystallized and shown (m.p. and mixed m.p.) to be largely ethyl 3α-hydroxy-6-oxo*allo*cholanate (see below).

ether, which was then used to dissolve the precipitate. The ether solution was washed with water, aqueous NH_3 and water; acidification of the washings gave 'ketonic acids' (0·156 g.). The dried (Na_2SO_4) ether was evaporated to give 'ketonic esters' (1·07 g.) as a partly crystalline solid. Repetition (twice) of the above process on the 'non-ketones' (17·00 g.) gave results as follows: 1st repetition: 'non-ketones' (16·77 g.); 'ketones' (0·045 g.). 2nd repetition: 'non-ketones' (16·43 g.); 'ketones' (0·02 g.). Hence, allowing for working losses, removal of the ketonic material from pig bile esters is almost completed by one Girard extraction; this finding is in contrast to observations on coypu bile acids (Haslewood, 1954*b*).

The first 'ketonic acids' (above) appeared to be impure 3α-hydroxy-6-oxo*allo*cholanic acid. Crystallization of the first 'ketonic esters' (1·07 g.) from ether gave a first crop (0·48 g., m.p. 135–136°). Evaporation of the filtrate left

Table 1. *Chromatography of ethylated pig 'bile acids'*

The 'bile acids' (8·7 g.) were fractionated on Al_2O_3 (100 g.). Each fraction was examined by paper chromatography as described in text. ECh = spot as ethyl chenodeoxycholate; EHd = spot as ethyl hyodeoxycholate. For details of unknowns X, Y and Z see text.

Fraction no.	Eluted by (solvent, ml.)	Wt. (g.) and appearance	Result of paper chromatography
I	Benzene, 320	1·75, oil	No spots
II	Benzene, 150	0·31, oil + crystals	No spots
III, IV	Benzene, 150 each	0·30, gum	ECh + EHd + unknown X
V	20% (v/v) Ether–benzene, 100	0·09, gum	
VI	As above	0·16, gum	
VII	50% (v/v) Ether–benzene, 100	0·23, gum	ECh + EHd only
VIII to X	Ether, 100 each	0·90, gum	
XI	Ether, 200	0·83, gum	
XII, XIII	Ether, 200 each	0·76, gum	
XIV	Ether, 400	0·47, gum	
XV	Ether, 400	0·31, crystals	
XVI	Ether, 600	0·30, gum	
XVII	Ether, 600	0·18, gum	ECh + EHd + ? unknown Y
XVIII	Ether, 600	0·04, gum	EHd + ? unknown Y
XIX	Ether, 600	0·03, gum	
XX, XXI	Acetone, 200 each	0·52, gum	EHd + ? unknown Y + unknown Z
XXII, XXIII	Ethanol, 200 each	0·86, gum	Unknown Z only
	Total eluted = 8·04 g.		

Fraction XV was crystallized from 25% (v/v) ether–light petroleum and thus gave short white needles of ethyl hyodeoxycholate (Haslewood, 1954*b*), m.p. 111–114°, which showed only one spot on paper chromatography. Hydrolysis of this yielded pure hyodeoxycholic acid, as glistening needles, m.p. 197–198°; $[\alpha]_D^{23} + 6{\cdot}9 \pm 0{\cdot}5°$ in ethanol (*c*, 2·1).

Girard separation. Pig bile acid 'esters' (18·7 g., prepared as under (*b*) above and carefully dried *in vacuo* over H_2SO_4) were boiled for 1 hr. under reflux with dry ethanol (100 ml.), acetic acid (10 ml.) and Girard's reagent 'T' (5 g.). The cooled mixture was diluted with ice water (3–4 vol.) and adjusted to pH about 7, with bromothymol blue as external indicator, by addition of 5N- and then 0·1N-NaOH. The mixture was extracted three times with ether; evaporation of the washed and dried (Na_2SO_4) ether left 'non-ketones' (17·00 g., after drying). The aqueous (ether-extracted) solution was left for some days after the addition of 10N-HCl (80 ml.). The (solid) precipitate was collected by filtration and the filtrate extracted twice with

a residue, which with 50% (v/v) light petroleum–ether gave a second crop (0·14 g., m.p. 110–126°) of crystals; evaporation of the final liquors gave a residual gum. The first crop (0·48 g.) of crystals was recrystallized from aqueous ethanol to give white glistening leaflets, m.p. 137·5–138·5°, of *ethyl 3α-hydroxy-6-oxoallocholanate.* (Found: C, 74·0; H, 10·1. $C_{26}H_{42}O_4$ requires C, 74·6; H, 10·0%.) Anchel & Schoenheimer (1938) give m.p. 136° but no analysis for this compound. This ester gave a colour on paper with the phosphomolybdic acid spraying reagent, but no spot after chromatography and spraying as described, with Bush's (1952) system *A*.

Simplified separation. The 'ethyl esters' (*n* g., where *n* was between 7 and 11) were dissolved in benzene (2–3*n* ml.) and poured on to a column (diam. 2·9 cm.) of Al_2O_3 (10*n* g.) made up in benzene. When the 'esters' had entered the column, they were eluted in the following order (fraction, approx. wt., solvent): fraction *A*, (0·35*n* g.), benzene (1 l.); fraction *B* (0·30*n* g.), ether (4 l.); fraction *C* (0·12*n* g.), acetone (500 ml.); fraction *D* (0·23*n* g.), ethanol

(500 ml.). Frequently a fraction (about 0·05*n* g.) remained on the column, presumably held as a salt, after hydrolysis by the Al_2O_3, and could be eluted with 1% (v/v) acetic acid–methanol. Fraction *A* was found to contain cholesterol and oily esters, fraction *B* was rich in ethyl chenodeoxycholate and contained ethyl hyodeoxycholate, fraction *C* was chiefly a mixture of ethyl hyodeoxycholate and ethyl hyocholate, and fraction *D* was very rich in ethyl hyocholate. Collected fraction *D* (3·8 g.) was boiled under reflux with ethanol (30 ml.) and 40% (w/v) aqueous KOH (4 ml) for 1 hr. The cooled solution was diluted with water and acidified with 5N-HCl. After being kept at 5°, the solid precipitate was collected, washed, dried by evaporation *in vacuo* with ethanol and crystallized from ethyl acetate. Yield, 1·7 g. of crude hyocholic acid (containing hyodeoxycholic acid) of m.p. about 190°. For further purification, this material was esterified with ethanol by process *A* (above). The ethyl esters (1·7 g.) were dissolved in a mixture of acetic acid (10 ml.) and acetic anhydride (2 ml.). To the solution, cooled in a beaker of water, was added slowly 8·5N-$HClO_4$ (0·1 ml.). The warm mixture was gently agitated for a few moments and then left for 10 min. It was then diluted with water and extracted twice with ether. The ether extract was washed with water, aqueous NH_3, water, dried (Na_2SO_4) and evaporated. The residue with light petroleum gave at once thin prismatic crystals of crude triacetyl ethyl hyocholate, which were collected and washed with cold light petroleum; yield, 1·5 g. The mother liquors gave on evaporation a gum which, with light petroleum, slowly crystallized as needles of impure diacetyl ethyl hyodeoxycholate (Haslewood & Wootton, 1950), m.p. about 90°. The weight of crude triacetyl ethyl hyocholate obtained by this process could be taken as a good guide to the percentage of hyocholic acid present in a mixture (mainly) of this substance and hyodeoxycholic acid, since the triacetyl ethyl ester crystallized very easily and was sparingly soluble in light petroleum. This method was used to determine the percentage of hyocholic acid in specimens of crude 'hyodeoxycholic' acid, and in crystalline material directly obtained after hydrolysis of bile salts by methods (*a*) and (*b*) described above. The average yield of purified hyocholic acid was approximately 5% of the 'bile acids'.

Hyocholic acid (II). Purified triacetyl ethyl hyocholate (0·4 g.) was boiled under reflux with ethanol (15 ml.) and 40% (w/v) aqueous KOH (1 ml.) for 35 min. The cooled solution was acidified with 5N-HCl, and the solid precipitate collected and washed. The liquors on standing gave white needles, m.p. 183–185°, of hyocholic acid, and the main precipitate, after drying, was crystallized from ethyl acetate to yield colourless short white needles, m.p. 188–189°, of *hyocholic acid* (II), $[\alpha]_D^{21} + 5{\cdot}5 \pm 0{\cdot}5°$ in ethanol (*c*, 1·7), $[M]_D \simeq +22°$. (Found: C, 70·3; H, 9·7%; equiv. wt. (titration) 410. $C_{24}H_{40}O_5$ requires C, 70·6; H, 9·8%; equiv.wt., 408.)

Properties of hyocholic acid. Hyocholic acid resembled hyodeoxycholic acid in appearance and crystallized in a similar way as white needles from acidified dilute aqueous solutions of its salts. From ethyl acetate, the pure acid formed crystals very similar to, but less glistening than, those of pure hyodeoxycholic acid. The m.p. of an approximately 50% (w/w) mixture of hyocholic acid (m.p. 188–189°) and pure hyodeoxycholic acid (m.p. 197–198°) was 184–196°; such mixtures, but with about 20% (w/w) of hyocholic acid, crystallized as apparently homogeneous needles and melted sharply at about 190°. Hyocholic acid gave a negative Hammarsten HCl test and an orange-red colour in the Liebermann–Burchard test. It was sparingly soluble in ether and benzene, dissolved fairly readily in acetone and warm ethyl acetate and easily in ethanol or methanol. A cold solution of hyocholic acid (20 mg.) in acetic acid (0·5 ml.) was treated with 20% CrO_3 (4 drops), added gradually with shaking. A similar solution of hyodeoxycholic acid (20 mg.) was treated in exactly the same way. Both CrO_3-treated solutions were diluted and extracted with ether. The (water) washed and dried (Na_2SO_4) ether extracts were evaporated, and each residue was dissolved in N-NaOH (0·5 ml.) with water (1·5 ml.). Each solution was yellow-orange in colour, that from hyocholic acid being about twice as dark as the other. The product from hyocholic acid, precipitated by acidification, could not be induced to crystallize and did not give a crystalline ethyl ester (process *A*); after Kishner–Wolff reduction of it, no crystalline material could be isolated.

Hyocholic acid (24 mg.) was dissolved in dry acetone (1 ml.) by warming, and the cooled solution left at about 23° for 16 hr. after addition of a saturated solution of HCl gas in dry ether (1 drop). Aqueous 10N-HCl (1 drop) was added and the solution left for 24 hr. No precipitate formed and evaporation of solvents at about 25° *in vacuo* left a residue which did not crystallize even when 'seeded' with hyocholic acid. Mild acetylation (acetic anhydride–pyridine at room temp.) gave no crystalline material. As starting material could not be recovered after this mild treatment, acetonide formation might have occurred, at least partially.

Derivatives of hyocholic acid

Ethyl hyocholate. This was prepared by process *A* and, crystallized from light petroleum–ether, formed short white needles, m.p. 76–78°. (Found, on a sample dried to const. wt. at 30°: C, 71·7; H, 10·1. $C_{26}H_{44}O_5$ requires C, 71·6; H, 10·1%.) Methyl hyocholate, similarly prepared, did not crystallize.

Triacetyl ethyl hyocholate. This was prepared by the $HClO_4$ method as described above (or, in inferior yield, by heating ethyl hyocholate for 2 hr. at about 95° in a mixture of equal vol. of acetic anhydride and pyridine), and when recrystallized from light petroleum–benzene formed large colourless crystals, very similar in appearance to those of cholesterol and having m.p. 185–187°; $[\alpha]_D^{22} + 27{\cdot}8 \pm 1°$ in ethanol (*c*, 1·2). (Found: C, 68·7; H, 9·0. $C_{32}H_{50}O_8$ requires C, 68·3; H, 8·9%.) Diacetyl ethyl hyodeoxycholate (Haslewood & Wootton, 1950) had $[\alpha]_D^{22} + 22{\cdot}3 \pm 1°$ in ethanol (*c*, 2·2). Triacetyl ethyl hyocholate was recovered unchanged after treatment in the cold with CrO_3 in acetic acid.

Triacetyl methyl hyocholate. This compound, similarly prepared and purified, resembled the corresponding ethyl ester in appearance. It had m.p. 184–185°; $[\alpha]_D^{22} + 28{\cdot}5 \pm 1°$ in ethanol (*c*, 1·1). (Found: C, 68·0; H, 8·8. $C_{31}H_{48}O_8$ requires C, 67·9; H, 9·0%.)

Diacetyl ethyl hyocholate (*probably ethyl 7α-hydroxy-3α:6α-diacetoxycholanate*, III). A solution of ethyl hyocholate (112 mg.) in dry pyridine (1 ml.) with acetic anhydride (1 ml.) was kept at about 23° for 16 hr. It was then diluted and extracted with ether. The ether was

washed with water, dried (Na_2SO_4) and evaporated. The residue was kept at about 5° with light petroleum or methanol, when it gradually formed large colourless cubical 'sugary' crystals (about 80 mg.); these were recrystallized from light petroleum–benzene and then gave (probably) *ethyl 7α-hydroxy-3α:6α-diacetoxycholanate* (III) of m.p. 121–123°, clearing at about 150°, depressed by ethyl 3α:6α-diacetoxy-7-oxocholanate (below). (Found: C, 68·85; H, 9·3. $C_{30}H_{48}O_7$ requires C, 69·2; H, 9·2%.) This compound was recovered substantially unchanged after: (i) keeping with freshly distilled toluene-*p*-sulphonyl chloride in pyridine or 2:4:6-collidine at about 20–25° for 4–5 days; or (ii) heating at 90–95° for 4 hr. with toluene-*p*-sulphonyl chloride in pyridine. Treatment of (III) with $POCl_3$ in pyridine gave unchanged material and gums; shaking with freshly sublimed PCl_5 in dry $CHCl_3$ with $CaCO_3$ gave a yellow product which could not be crystallized before or after treatment with H_2 and Adams Pt catalyst.

Ethyl 3α:6α-diacetoxy-7-oxocholanate. A solution of the above diacetate (III, 120 mg.) in acetic acid (1 ml.) was treated with 20% CrO_3 (0·1 ml.), added slowly with cooling and mixing. After 10 min. the product was diluted and extracted with ether. The ether was washed, dried (Na_2SO_4) and evaporated. The residue (115 mg.), which was recovered quantitatively in the 'non-ketonic' fraction after treatment with Girard's reagent 'T' in the usual way, was crystallized by the addition of light petroleum and recrystallized from light petroleum–benzene. *Ethyl 3α:6α-diacetoxy-7-oxocholanate* thus gave groups of small white needles (81 mg.), m.p. 121–123°. (Found: C, 69·2; H, 8·9. $C_{30}H_{46}O_7$ requires C, 69·5; H, 8·9%.) Three attempts by methods found successful in other cases were made to reduce this substance by the Kishner–Wolff process. All failed to yield crystalline material at any stage, and paper chromatography of the (ethylated) acids recovered from these experiments failed to give convincing evidence for the formation of hyodeoxycholic acid in them. A spot running rather faster than ethyl hyodeoxycholate was noticed, and an unsuccessful attempt to isolate an identifiable substance responsible for this was made by separation on paper, as previously applied (Haslewood, 1954*b*).

Clemmensen reduction: the ketone (0·1 g.) was added to a flask containing freshly amalgamated zinc wool (1 g. of Zn), water (0·75 ml.) and 10N-HCl (1·0 ml.). The flask was gently heated, under reflux, for 5·5 hr., during which time 10N-HCl (total 2·5 ml.) was added down the condenser at the rate of about 0·5 ml. every 30 min. The diluted cooled solution was extracted with ether and the ether washed with water, aqueous NH_3, water, dried (Na_2SO_4) and evaporated: the residue (59 mg.) was 'neutrals'. Acidification of the aqueous NH_3 washings gave 'acids' (19 mg.). Attempts were made to crystallize the (hydrolysed) 'neutrals' and also the 'acids' from ethyl acetate, but without success. Each fraction was then re-ethylated (process *A*) and examined by paper chromatography. The ethylated 'acids' gave no spots, but the re-ethylated 'neutrals' gave faint spots corresponding to ethyl hyocholate and ethyl hyodeoxycholate. The re-ethylated 'neutrals' (53 mg.) were dissolved in benzene, and the solution was poured on to Al_2O_3 (0·5 g., Hopkin & Williams Ltd., neutralized as described by Shoppee, 1949) in a column. Benzene (30 ml.) eluted a fraction (39 mg.), which crystallized from light petroleum and then from aqueous ethanol as short needles, m.p. 150–157°, tetranitromethane in $CHCl_3$, no colour. (Found: C, 75·0; H, 10·7. $C_{26}H_{44}O_4$ requires C, 74·3; H, 10·5%.) The nature of this material has not been further elucidated. No further crystalline substance could be isolated in this experiment. A second Clemmensen reduction, with acetic acid as solvent, gave, after similar processing, no crystalline material.

Hydrogenation: the ketone (40 mg.) in acetic acid (5 ml.) containing H_2SO_4 (0·2 ml.) was shaken with PtO_2 (50 mg.) in an atmosphere of H_2 at slightly increased pressure for 4 hr. Extraction of the product from the filtered and (aqueous) diluted mixture with ether gave, on evaporation of the washed ether, a residue, which was acetylated by the $HClO_4$ method. The only crystalline substance recovered after this process was an impure sample of triacetyl ethyl hyocholate.

3α:6α-Dihydroxy-7-oxocholanic acid (acid A, IV). The above ketone (88 mg.) was hydrolysed by boiling under reflux with a mixture of ethanol (2 ml.) and 40% (w/v) aqueous KOH (0·2 ml.). The cooled solution was diluted with water, acidified with HCl and treated with NaCl (excess). The solid precipitate was collected, washed with water and evaporated to dryness with ethanol *in vacuo*. The residue crystallized on adding ether, and the crystals were collected and washed with cold ether. Recrystallization from aqueous ethanol or light petroleum–ethanol gave *3α:6α-dihydroxy-7-oxocholanic acid* (acid A, IV) as white needles which decomposed at about 211–215°, according to the rate of heating; $[\alpha]_D^{23} + 12{\cdot}3 \pm 1°$ in ethanol (*c*, 0·8), $[M]_D \simeq +50°$. (Found: C, 70·6; H, 9·4. $C_{24}H_{38}O_5$ requires C, 70·9; H, 9·3%.)

(Probably) 3α:6α:7α:24-tetrahydroxycholane, and its acetates. Triacetyl ethyl hyocholate (0·2 g.) with dry ether (20 ml.) and powdered $LiAlH_4$ (0·2 g.) were gently heated under reflux for 2 hr. The mixture was poured onto ice, in aqueous HCl. The organic product was immediately extracted with ether and the ether washed with water, dried (Na_2SO_4) and evaporated. The (neutral) residue (131 mg.) formed gels with organic solvents, but crystals of m.p. about 140° were formed with light petroleum–ether; these were not further purified.

Tetraacetate: a solution of the $LiAlH_4$ product (64 mg.) in acetic acid (1 ml.) with acetic anhydride (0·2 ml.) was treated with 8·5N-$HClO_4$ (1 drop). After 10 min., the mixture was diluted with water and the separated solid collected, washed and dried with ethanol *in vacuo*. With light petroleum small white prisms appeared; these were recrystallized from dilute ethanol to give (probably) *3α:6α:7α:24-tetraacetoxycholane*, m.p. 173–175°. (Found: C, 68·3; H, 9·1. $C_{32}H_{50}O_8$ requires C, 68·3; H, 8·9%.)

Triacetate: the $LiAlH_4$ product (68 mg.) was dissolved in dry pyridine (0·5 ml.) with acetic anhydride (0·5 ml.) and the solution kept at about 23° for 16 hr. It was then diluted with approx. N-HCl (excess) and the precipitate collected, washed and dried by evaporation *in vacuo* with ethanol. The residue crystallized with light petroleum as needles (70 mg.) of m.p. 115–118°; these were recrystallized from aqueous ethanol to give long white needles of (probably) *7α-hydroxy-3α:6α:24-triacetoxycholane*, m.p. 114–116°; $[\alpha]_D^{20} + 22{\cdot}5 \pm 1°$ in ethanol (*c*, 0·9). (Found: C, 68·7; H, 9·2. $C_{30}H_{48}O_7$ requires C, 69·2; H, 9·2%.) An attempt was made to dehydrate this substance (62 mg.) with freshly distilled $POCl_3$ (0·3 ml.) in dry pyridine (1 ml.) for 22 hr. at about 23°. No crystalline material could be isolated from the product, after careful chromatography on Al_2O_3.

3α:6α:24-*Triacetoxy-7-oxocholane.* The triacetate (30 mg.) in acetic acid (0·5 ml.) was treated at about 20° with 20% CrO_3 (2 drops). After 10 min., with occasional shaking, the solution was diluted with water and the solid precipitate collected, washed and dried by evaporation with ethanol *in vacuo*. The residue was crystallized from light petroleum and then from aqueous ethanol to give long white glistening needles of 3α:6α:24-*triacetoxy-7-oxocholane*, m.p. 130–131°; $[\alpha]_D^{19} + 21{\cdot}3 \pm 1°$ in ethanol (*c*, 0·75). (Found: C, 69·7; H, 9·1. $C_{30}H_{46}O_7$ requires C, 69·5; H, 8·9%.) This substance (194 mg.) was added to a suspension of powdered $LiAlH_4$ (0·2 g.) in dry ether (20 ml.) and the mixture was gently boiled under reflux for 2 hr. The $LiAlH_4$ product was worked up as described above and was then acetylated by the $HClO_4$ method and separated by chromatography. The above tetraacetate (110 mg.) and an impure sample (about 5 mg.) of a different compound could be isolated. 3α:6α:24-Triacetoxy-7-oxocholane (10 mg.) was gently heated under reflux at intervals for 5 days with 50% (v/v) ethanol–water (5 ml.), semicarbazide hydrochloride (0·1 g.) and hydrated sodium acetate (0·1 g.). A few crystals, decomp. about 260°, were formed, but these were also obtained with semicarbazide and sodium acetate alone. Dilution of the reaction mixture (after 5 days) gave crystals (5–8 mg.) of m.p. about 120°, which did not contain nitrogen. An attempt at Kishner–Wolff reduction of the above ketone (39 mg.) gave a product (32 mg.) from which, after acetylation and chromatography, no identifiable material could be isolated.

Thilobilianic acid (V). A solution of ethyl hyocholate (0·2 g.) in acetic acid (2 ml.) was kept at about 20° by cooling in water and treated, with shaking, with 20% CrO_3 (1 ml.) added gradually from a burette during 5 min. After 3·5 hr. at 20°, with occasional shaking, the solution was diluted and shaken twice with ether. Insoluble material which separated was dissolved in aqueous NH_3, which was used to extract the ether. The ether was then washed with water and all the aqueous NH_3 and water washings were combined and treated with 5N-HCl (excess) and NaCl (excess). The mixture was warmed gently and surplus ether was expelled by blowing N_2 gas on to the surface. The gummy solid which separated was collected, washed and stirred with dilute aqueous $NaHCO_3$. The filtered solution was acidified with HCl and the precipitate collected, washed with water and dried by evaporation *in vacuo* with ethanol, giving gummy 'acids' (120 mg.). Evaporation of the above washed ether gave 'neutrals' (32 mg.); this material could not be induced to crystallize, and treatment of it by the Kishner–Wolff method gave no crystalline product.

The 'acids' (120 mg.) were transferred in ethanol to a small metal bomb and solvent was removed with N_2. The residue was dissolved in ethanol (4 ml.), to which was added sodium (0·25 g.) and '100%' hydrazine hydrate (0·4 ml.). The bomb was sealed and heated at 190–200° for 4 hr., and then cooled. The contents were diluted with water. Acidification of the filtered solution gave a white solid, which was collected, washed with water and dried by evaporation *in vacuo* with ethanol. The residue was crystallized from ethyl acetate to give thilobilianic acid (V) as small white silky needles, m.p. 255–257° (decomp.). (Found: C, 68·0; H, 8·8%; equiv.wt. (titration) 141. Calc. for $C_{24}H_{38}O_6$: C, 68·6; H, 9·05%; equiv.wt. 144.) Yield, about 25 mg. Wieland & Dane (1932) give m.p. 260–262° (decomp.) for this substance.

Preparation of reference compounds

3α:6α-*Dihydroxy-7-oxocholanic acid* (*acid A*, IV). Ethyl 3α-acetoxy-7-oxocholanate (Haslewood, 1954*b*; from 0·1 g. of 3α-hydroxy-7-oxocholanic acid) was dissolved at about 20° in 1 ml. of a freshly made solution of bromine (0·1 ml.) in acetic acid (7·8 ml.). To this was added 1 drop of a solution of HBr in acetic acid, and the stoppered mixture was left at about 20° for 16 hr.; bromine disappeared. Solvent was evaporated at about 30° *in vacuo*, and to the residue were added KOH (approx. 0·3 g.) and methanol (4 ml.). The solution was gently boiled under reflux for 1 hr., and was then cooled, diluted with water and acidified with HCl. Addition of NaCl (excess) gave a solid which was collected, washed with water and evaporated with ethanol to dryness *in vacuo*. A solution of the residue in ethyl acetate was filtered and evaporated to about 1 ml. Crystals separated in two forms: needles (m.p. 201–206° decomp.) and large prisms (m.p. about 225°). The needles, which were in much larger amount, were collected separately and recrystallized from light petroleum–ethyl acetate. Needles, decomp. range about 211–214°, were obtained, and these did not depress the m.p. of a sample of (IV) from hyocholic acid (above). A sample (1 mg.) of (IV) from each source was methylated with diazomethane in ether and solvent removed. The residues were examined by infrared spectroscopy by Dr I. D. P. Wootton, who reported that the spectra were identical. Evaporation of solvent (CS_2) used for the infrared analysis left crystalline samples of the methyl esters; the crystals were washed with light petroleum. The crude products melted as follows: from hyocholic acid, at 110–115°; from 3α-hydroxy-7-oxocholanic acid, at 114–117°; mixed m.p. 110–115°. The substance (IV) was also obtained (by bromination and alkaline hydrolysis of the product) from 3α-acetoxy-7-oxocholanic acid, prepared *in situ* by acetylation of 3α-hydroxy-7-oxocholanic acid.

3α:6α:24-*Triacetoxycholane* (VI). To a solution of ethyl hyodeoxycholate (0·1 g.) in dry ether (10 ml.) was added powdered $LiAlH_4$ (0·1 g.), and the mixture was gently boiled under reflux for 1 hr. It was then cooled and poured into HCl–ice. The organic material was extracted with ether and the ether washed with water, aqueous NH_3, water, dried (Na_2SO_4) and evaporated. The residue, from light petroleum–ether, gave large crystals of m.p. about 170°, which appeared to be hydrated. (Found: C, 75·4; H, 11·1. $C_{34}H_{42}O_3$ requires C, 76·2; H, 11·1%.) This material (30 mg.) was dissolved in pyridine (1 ml.) with acetic anhydride (1 ml.) and the mixture kept at about 25° for 24 hr.; it was then diluted with aqueous HCl (excess). The precipitate was collected, washed with water, evaporated to dryness with ethanol *in vacuo* and crystallized twice with light petroleum–benzene, giving white needles of 3α:6α:24-*triacetoxycholane* (VI), m.p. 129–131°. (Found: C, 71·1; H, 9·5. $C_{30}H_{48}O_6$ requires C, 71·4; H, 9·5%.)

Ethyl 3β:6α-*dihydroxycholanate.* 3β:6α-Dihydroxycholanic acid, prepared from pig gallstone (Haslewood & Wootton, 1950), was ethylated by process *A*: the product crystallized readily from light petroleum–benzene in short white needles of *ethyl* 3β:6α-*dihydroxycholanate*, m.p. 131–134°. (Found: C, 74·6; H, 10·2. $C_{26}H_{44}O_4$ requires C, 74·3; H, 10·5%.) This substance did not give a spot on paper chromatography as described with Bush's (1952) system *A*.

DISCUSSION

Chemical. The present proof of the structure of hyocholic acid rests largely on the correctness of formula (IV) for the acid A, obtained by alkaline hydrolysis of brominated 3α-acetoxy-7-oxocholanic acid or its ethyl ester.

Wieland & Dane (1932) and Dane & Wulle (1941) brominated 7-oxocholanic acid. They obtained a product in which the bromine atom was shown to be at C-6 and probably β-orientated. Lardon (1947) brominated methyl 3α-succinyloxy-7-oxoaetianate and heated the crude product with methanolic sodium hydroxide. The acid so obtained was converted into a substance described as methyl 3α:6α-diacetoxy-7-oxoaetianate; Kishner–Wolff reduction of this, followed by further treatment, gave methyl 3:6-dioxo*allo*aetianate in poor yield. More recently, Takeda, Igarashi & Komeno (1954*a*) actually brominated methyl 3α-acetoxy-7-oxocholanate. The principal product was resistant to debromination and hence the bromine atom (at C-6) was probably β-orientated (*cis* elimination as HBr with H at C-5 very difficult). Hot saponification (Takeda, Komeno & Igarashi, 1954*b*) of the bromo derivative gave an acid having m.p. 227° which may well be identical with acid A (IV), for the m.p. (decomposition point) of A is variable, according to the rate of heating. The acid of m.p. 227° is thus probably (IV), Walden inversion at C-6 having occurred, as expected, during hydrolysis. Takeda *et al.* (1954*b*) suggested 3α:7-dihydroxy-6-oxo*allo*cholanic acid for the structure of their acid, but provided no evidence for this formula. By cold saponification of their (presumably) methyl 3α-acetoxy-6β-bromo-7-oxocholanate, Takeda *et al.* derived an acid of m.p. 185°, to which they assigned formula (IV) and which they converted into hyodeoxycholic acid; an explanation of these findings is not at present obvious.

This prior work tends on the whole to support the structure (IV) for acid A.

Thilobilianic acid (V) was made by Wieland & Dane (1932) by oxidation of 7-hydroxy- and 7-oxo-cholanic acids, and may be assumed to have the hydrogen atom at C-5 in the β position; hence its formation from hyocholic acid proves that this substance too has the 5β (cholane) structure.

Examination of molecular models suggests that the reactions of a 7α-hydroxyl group in a cholane compound would be hindered, and that such hindrance would be greatly increased by a hydroxyl or acetoxy group at C-6α. 7α-Hydroxylation also might be expected on biogenetic grounds, in the present case. Such considerations do not, however, amount to proof of configuration.

Hyocholic acid is thus satisfactorily proved to be a 3α:6α:7-trihydroxy- and is probably 3α:6α:7α-trihydroxy-cholanic acid (II); the 'hindered' group is the one at C-7.

This appears to be the first authentic example of a 6:7-dihydroxycholane, although 6-hydroxy-7-oxo and 7-hydroxy-6-oxo compounds of this type are well known. The remarkable feature of the 6:7-dihydroxycholane now discovered is the enhancement of the (somewhat) hindered nature of the C-7-hydroxyl group by the neighbouring C-6α-hydroxyl (or -acetoxy) group. This C-7 group was more difficult to acetylate than a C-12α-hydroxyl group and could not (when –OH at C-6 was acetylated) be induced to give a toluene-*p*-sulphonyl derivative. The corresponding C-7 ketone (acetoxy at C-6; >C=O at C-7) was entirely inert to Girard's reagent and semicarbazide.

It is of course well known that a C-7α-hydroxyl group in cholane derivatives is a little hindered in esterification reactions, and that the corresponding ketone, although it forms a semicarbazone (Haslewood, 1943), does not quantitatively react with Girard's reagent (Hoehn, Schmidt & Hughes, 1944; Haslewood, 1954*b*); nevertheless, the increased degree of 'hindering' at C-7 observed in hyocholic acid and its derivatives was quite unexpected and accounted for much of the difficulty of the present investigation.

Although its conjugate was not isolated from paper chromatograms, the general behaviour of hyocholic acid and its esters leaves little doubt that its glycine conjugate was in fact the substance observed by Haslewood & Sjövall (1954) and mentioned above.

Trickey's (1950) acid '$C_{27}H_{44}O_6$' was isolated only as the azoylamide of indefinite melting point; his formula, based only on elementary analysis, might be explained if his material was hydrated hyocholic acid azoylamide. The percentage (6 %) of pig bile acids in this fraction would agree with the findings for hyocholic acid now recorded. The point is an important one, as no mammal has so far been found to secrete a C_{27} bile acid: it would therefore be of great interest if the occurrence of such a compound could be confirmed.

Biological. As a working hypothesis, hyocholic acid in the pig can be considered as corresponding to cholic acid in other mammals, i.e. where these have 3α:7α:12α-trihydroxy-cholanic (cholic) acid, the pig has 3α:6α:7α-trihydroxycholanic (hyocholic) acid.

The dog can make cholic acid from cholesterol, and the rat has been shown to make its chief bile acids (cholic and chenodeoxycholic) from the same source (for references, see Haslewood, 1955). The rabbit converts cholesterol into deoxycholic (3α:12α-dihydroxycholanic) and cholic acids (Ekdahl & Sjövall, 1955). Similarly, the pig may make from cholesterol the bile acids mentioned (above) as occurring in its bile.

The pig is not known to form deoxycholic or cholic acids, or any bile acid hydroxylated at C-12. No other mammal has so far been shown to make (normally) any bile salt hydroxylated at C-6; the only known examples of creatures having this ability are certain teleostean fish and the domestic hen (Ohta, 1939; Isaka, 1940; Isaka & Azato, 1940; Mabuti, 1941; Yamasaki, 1951).

Thus pig bile salts are unique in a special way. This uniqueness must be due to (*a*) mutation occurring in ancestors having already the cholesterol → cholic acid sequence (or part of it), or (*b*) descent from a very primitive form originally making C-6-hydroxylated bile salts. A decision between possibilities (*a*) and (*b*) obviously cannot be made except by consideration of all the available evidence, but when the evolutionary position of the pig is ascertained its relationship to that of other vertebrates will have to account for the remarkable chemical nature of pig's bile salts. In considering this question, it should be noted that the general trend of the present work suggests that the chemical nature of bile salts is of a very conservative character.

SUMMARY

1. Hyocholic acid, $C_{24}H_{40}O_5$, from pig bile has been shown to be 3α:6α:7-trihydroxycholanic acid (II); the hydroxyl group at C-7 is probably α-orientated.

2. This substance appears to be the first authentic example of a 6:7-dihydroxycholane: the presence of the hydroxyl group at C-6 in this cholane derivative causes marked hindrance in the reactions of the C-7 hydroxyl group and of the ketone derived from it.

3. Pig bile contains at least one unidentified substance, giving a spot on paper chromatograms of the ethylated 'bile acids'.

4. Attention is drawn to the remarkable chemical nature of pig bile salts, and it is suggested that this unique nature must be taken into consideration in studies of pig evolution.

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Experiments on the Lysine and Aspartic Acid Residues in Bacitracin A

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In several structural features the bacitracin family appears to be unique among polypeptides that have so far been subjected to detailed chemical study. The *N*-terminal and sulphur-containing residues in bacitracin A have been discussed in an earlier paper (Lockhart, Abraham & Newton, 1955). In addition to this portion of the molecule, the lysine residue is a centre of interest.

The earliest experiments on the reaction of bacitracin A with 1-fluoro-2:4-dinitrobenzene (FDNB) revealed that neither of the two amino groups of the lysine residue in the molecule was free (Craig, Hausmann & Weisiger, 1952; Newton & Abraham, 1953). Studies of the amino acid sequence in bacitracin A showed that the probable arrangement of residues in the vicinity of the lysine was