Metabolism of Steroids

5. KETONIC DERIVATIVES OF CHOLIC ACID FROM COWS' BILE

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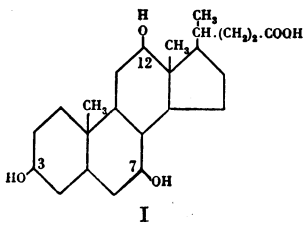
The only ketocholanic acids isolated from bile, apart from those obtained in experiments involving the giving of bile acid derivatives to animals, have been 3-hydroxy-12-ketocholanic acid from ox bile (Wieland & Kishi, 1933), a 3-hydroxy-6-ketocholanic acid from hog bile (Fernholz, 1935), and 3-hydroxy-7-ketocholanic acid from guinea-pig bile (Imai, 1937). Hughes (1942) states that ketonic acids were only present, if at all, in traces in the ox bile examined by her, and Berman, Snapp, Hough & Ivy (1940) used a cattle bile in which they could detect no 'keto reacting substances'. However, in this more recent work, only small amounts of bile were used, and it was considered worth while to examine larger amounts of fresh bile for ketonic acids. In order to interpret the results obtained, a comparison had to be made between the six possible ethyl hydroxy-ketocholanates with the cholic acid configuration. This work in turn made possible some further observations concerning the valuable Hammarsten test for cholic acid derivatives.

EXPERIMENTAL

General methods. Fresh cows' bile was worked up by the method of White (1929). After removal of neutral substances, the acids were fractionated and esterified with diazoethane. Ketonic esters were separated with Girard's reagent and purified by adsorption on alumina. The resulting crystalline esters were finally identified by comparison with those given in Table 1. All melting-points are uncorrected. Elementary micro-analyses were by Mr A. T. Macdonald, Edinburgh.

Abbreviations. (*a*) In the following, only cholanic acid derivatives substituted at C_3, C_7 and C_{12} are mentioned, and cholic acid (I) is therefore abbreviated to (II); the formulae of ketonic acids and esters being similarly written.



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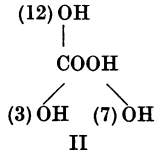


II

(*b*) Process A, applied to purification of ethyl esters consisted in each case of dissolving the ester in benzene and running the solution through a column of Al_2O_3 (Hopkin & Williams, 10 g./g. of ester in a narrow cylindrical tube). The ester was then eluted with portions of benzene; these were collected separately and evaporated. If the residue gave crystals with l.p. (light petroleum, b.p. 40–60°), these were collected and recrystallized from l.p.-benzene.

(*c*) H-test = Hammarsten HCl test, as described (Haslewood, 1943).

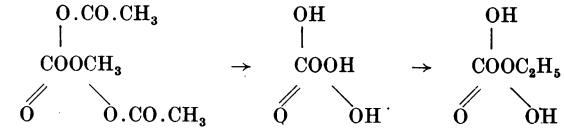
(*d*) 20% CrO_3, as described (Haslewood, 1944).

Isolation of esters from bile. Fresh cows' bile from the slaughter-house was treated with 60 g. of NaOH and 100 ml. of water per litre of bile and the mixture heated, with continual agitation, on the boiling water-bath for 20 hr. The hot product was made almost neutral to phenolphthalein with 10N-HCl, then cooled and strongly acidified with 10N-HCl. After 24 hr., the precipitate was collected, washed and kneaded with water, and dissolved in 100 ml. of 2·5N-NaOH/l. of bile. The solution was treated with acetic acid and NaOH until it was just alkaline to phenolphthalein and 20 g. charcoal per litre of bile was stirred into the hot mixture in a large dish. The product was evaporated on the water-bath and the residue dried in an oven at 70°. Yields: 230 and 101 g. of charcoal-product from 2670 and 1100 ml. of cows' bile respectively. The total charcoal-product (331 g.) was powdered and continuously extracted in a Soxhlet-type apparatus with ether until the extract was colourless. Evaporation of the ether gave *CB Neutrals I* (0·8 g.). Extraction was now carried out with acetone, giving *CB Neutrals II* (0·7 g.). Finally, extraction was done with ethanol, which removed most of the material from the charcoal. The ethanol extract was diluted with 4–5 vol. of water and continuously extracted with ether, giving *CB Neutrals III* (0·95 g.). Complete examination of the neutral material (2·45 g.), which contained cholesterol (approx. 0·75 g.), as well as, apparently, some 7-hydroxycholesterol, was suspended after the publication of Pearlman (1944), who used much larger amounts of bile. The ether-extracted aqueous solution was heated to expel ether, and acidified with HCl; the precipitate, washed and dried *in vacuo* over H_2SO_4, weighed 104 g. This was dissolved in 208 ml. of 2N-NaOH and the solution diluted to 500 ml. with water, heated on the water-bath, neutralized to phenolphthalein with acetic acid, further diluted to 1 l. and treated with 150 ml. of $MgCl_2$ solution (approx. 20 g./100 ml.). The mixture, made just alkaline to phenolphthalein, was heated on the water-bath for 16 hr. and left at *c.* 20° for 16 hr. The large precipitate was collected, washed, and partially dried; it was then stirred and left for about 70 hr. with dilute HCl (excess). The insoluble product was collected, washed, and dried *in vacuo* over H_2SO_4, giving *CB Mg-insol.* (48 g.). The $MgCl_2$ liquors, on acidification with HCl, gave a precipitate which, after washing and drying, was *CB Mg-sol.* (55 g.). This product was extracted with successive quantities of boiling ethyl acetate. The first litre of this solvent extracted 46 g. of material, from which

crystallized 36·4 g. of cholic acid, and, after evaporation, a second crop of 1·2 g. The liquors, on evaporation, left *CB ethyl acetate-sol. I* (8·4 g.). A second extraction of CB Mg-sol. with boiling ethyl acetate (*c.* 2 l.) gave *CB ethyl acetate-sol. II* (7·4 g.). The residue was *CB ethyl acetate-insol.* (1·45 g.). CB ethyl acetate-sol. I (8·4 g.) was dissolved in ethanol and treated with excess of diazoethane. After standing overnight, the diluted solution was extracted with ether. The extract, washed with water, dilute H_2SO_4, water, NH_3 solution, and water was dried ($CaCl_2$) and evaporated. The residual esters formed a yellow oil (8·8 g.) which was dried *in vacuo* at 100° and dissolved in dry ethanol (40 ml.) with pure acetic acid (4 ml.) and Girard's reagent T (2·2 g.). The solution was refluxed, with a condenser carrying a $CaCl_2$ trap, for 2 hr., and was then cooled, diluted with 2–3 vol. of ice-water and neutralized to bromothymol blue with 5N-NaOH (*c.* 12 ml.). Five ether extractions now gave, on evaporation of the washed and dried ether, 8·0 g. of non-ketones, while the aqueous solution yielded, after standing for 16 hr. with the addition of 10N-HCl (25 ml.), an oil which was extracted with ether. Evaporation of the washed and dried ether left the ketonic esters as a fragrant gum (0·2 g.). This material was dissolved in benzene (*c.* 40 ml.) and the solution was allowed to run through a column of Al_2O_3 (Hopkin & Williams, 2 g.) of diam. 0·8 cm. The column was eluted by washing first with benzene and finally with ether, and fractions collected as follows: (I) original solvent + 10 ml. benzene, oil, 0·1 g.; (II) 20 ml. benzene, gum 0·02 g.; (III) 100 ml. benzene, gum <5 mg.; (IV) 50 ml. ether, gum 0·08 g. Fraction II, with 50% v/v l.p./ether gave crystals which, after recrystallization from dilute ethanol gave *ester* K_1 (1–2 mg.), m.p. 176–178°, corresponding in m.p. and H-test to ethyl-7:12-dihydroxy-3-ketocholanate (below) and also to a specimen isolated in a similar manner from fresh, mixed 'ox bile'. (Found: C, 72·1; H, 9·3. Calc. for $C_{26}H_{42}O_5$: C, 71·9; H, 9·7%.) Ester K_1 was hydrolyzed to an acid which on CrO_3 oxidation was converted into (partially purified) dehydrocholic acid. Fraction IV similarly gave *ester* K_2 (20 mg.), m.p. 154–158°, not depressed by ethyl 3:12-dihydroxy-7-ketocholanate, and giving a similar colour in the H-test. (Found: C, 71·3; H, 9·2. Calc. for $C_{26}H_{42}O_5$: C, 71·9; H, 9·7%.) Mixed m.p. with all other esters in Table I gave large depressions. Ester K_2 (4 mg.) in acetic acid (4 drops) with 20% CrO_3 (1 drop) gave, after dilution, a solid which from dilute ethanol formed white needles, m.p. 220–222°, decomp., not depressed by ethyl dehydrocholate (m.p. 219–220°, decomp.).

By treatment similar to the above, CB Mg-insol. (48 g.) gave ethyl esters (44·5 g.), separated into non-ketones (44 g.) and ketones (0·2 g.); this ketonic material yielded a further sample of ester K_2 (10 mg.), m.p. 156–157°. CB ethyl acetate-sol. II gave no identifiable ketonic material.

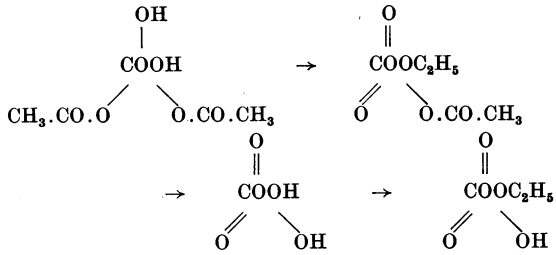
Ethyl 7:12-*dihydroxy*-3-*ketocholanate*. The route to this substance was:



Methyl 7:12-diacetoxy-3-ketocholanate (0·5 g., Haslewood, 1944) was boiled for 40 min. with approx. N-NaOH (10 ml. in 50% v/v ethanol-water). The cooled diluted mixture was filtered, saturated with NaCl and acidified with H_2SO_4. The precipitated acid was collected after 48 hr.: it was washed, dissolved in ethanol and the solution saturated with diazoethane. After 16 hr. the mixture was diluted and treated with excess $NaHCO_3$. The crystalline undissolved material was collected, washed, and recrystallized from dilute ethanol; it formed white leaflets (0·15 g.), m.p. 176–178°, not depressed by K_1. By process A, the ester yielded long white needles of final m.p. 181–182°. (Found: C, 71·7; H, 9·5. $C_{26}H_{42}O_5$ requires C, 71·9; H, 9·7%.) In the H-test, this ester and K_1 gave yellow → green → violet colours, and both substances showed violet colours with *m*-dinitrobenzene, ethanol, and alkali.

Ethyl 3:7-*dihydroxy*-12-*ketocholanate*. A mixture of crude 3:7-dihydroxy-12-ketocholanic acid (0·45 g., Wieland & Kapitel, 1932), ethanol (5 ml.) and H_2SO_4 (0·5 ml.) was refluxed for 2·5 hr. The cooled diluted product was treated with Na_2CO_3 (excess) and the precipitated solid was collected and recrystallized from dilute ethanol (charcoal). Yield, 0·25 g., m.p. 161–164°, giving, by Process A, long white needles, m.p. 161–162°. (Found: C, 72·2; H, 9·7. $C_{26}H_{42}O_5$ requires C, 71·9; H, 9·7%.) H-test, negative, *m*-dinitrobenzene test, negative.

Ethyl 7-*hydroxy*-3:12-*diketocholanate*. The route was:



12-Hydroxy-3:7-diacetoxycholanic acid (2·3 g., m.p. 259–261°, Wieland & Kapitel, 1932) was refluxed with ethanol (46 ml.) and 10N-HCl (4·6 ml.) for 2 hr. After dilution, the cold mixture was left for 16 hr. and the liquor then decanted from the partially crystalline precipitate, which was washed, dissolved in acetic acid (20 ml.) and treated with 20% CrO_3 (4 ml.). After 15 min., the solution was diluted, and after 48 hr. the precipitate was collected, washed and dissolved in benzene, which was dried with $CaCl_2$, filtered and evaporated. The residue, from l.p.-ether, gave *ethyl* 7-*acetoxy*-3:12-*diketocholanate* (0·55 g.) of m.p. 161–164°, raised to 168–168·5° by Process A. (Found: C, 70·7; H, 9·0. $C_{28}H_{42}O_6$ requires C, 70·9; H, 8·9%.) This substance (0·5 g., m.p. 161–164°) with NaOH (5 ml. of approx. N/2 in 90% (v/v) ethanol-water) was boiled under reflux for 20 min. The cooled product was diluted, acidified with HCl and saturated with NaCl. After 16 hr., the precipitate was collected, washed, and evaporated until dry with ethanol *in vacuo*. The residue in ethanol (5 ml.), saturated with HCl gas was left for 48 hr., after which the solution was diluted and treated with Na_2CO_3 (excess). The solid undissolved material was collected, washed and evaporated with ethanol to dryness *in vacuo*. The residue was boiled with ether and charcoal, and the filtered ether evaporated. The residue with l.p.-ether gave crystals (0·35 g.) of m.p. *c.* 136–140°: these were impure, for by Process A this product was converted into white needles of m.p. 167–168°; H-test, negative, *m*-dinitrobenzene test, positive. (Found: C, 72·0; H, 9·2. $C_{26}H_{44}O_5$ requires C, 72·2; H, 9·3%.)

DISCUSSION

Table 1 shows properties of the ethyl esters of the six possible hydroxy-keto acids with the steric configuration of cholic acid. It is clear from this table and from the experimental section that the esters now isolated from cows' bile correspond to 7:12-dihydroxy-3-keto- and 3:12-dihydroxy-7-keto-cholanic acids. The experimental conditions excluded post-mortem bacterial action in the bile, and in a separate experiment it was found that cholic acid did not undergo the kind of 'autoxidation' demonstrated by Bergström & Wintersteiner (1942) for cholesterol. There seems no reason to doubt that the keto-acids could be formed by bacteria in the cows' intestines, and, if this is the case, subsequent absorption and re-excretion would account for their presence in the bile. The bacteria concerned would probably include organisms other than *Alcaligenes faecalis*, for Schmidt, Hughes, Green & Cooper (1942) state that this organism probably does not form a dihydroxy-3-ketocholanic acid, although it does convert cholic acid to the 7-keto derivative (Hoehn, Schmidt & Hughes, 1944). A further study of Table 1 indicates that a positive (yellow) Hammarsten test may be given by cholic acid derivatives when the conditions stated by Yamasaki, Takahashi & Kim (1939) for the production of a blue or violet colour in the test are modified by the presence of a keto group at C_7 in the cholane nucleus.

Table 1. *Esters of hydroxyketocholanic acids*

Ester	m.p.	Response to Hammarsten test	Reference
Ethyl 7:12-dihydroxy-3-ketocholanate	181–2°	Yellow → green → violet	New substance
Ethyl 3:12-dihydroxy-7-ketocholanate	160–1°	Yellow	Haslewood (1943)
Ethyl 3:7-dihydroxy-12-ketocholanate	161–2°	Negative	New substance
Ethyl 3-hydroxy-7:12-diketocholanate	153–4°	Negative	Borsche & Hallwass (1922)
Ethyl 7-hydroxy-3:12-diketocholanate	167–8°	Negative	New substance
Ethyl 12-hydroxy-3:7-diketocholanate	168–9°	Yellow	Haslewood (1944)

SUMMARY

1. Ethyl esters isolated from fresh cows' bile indicate that the bile contains very small amounts (of the order of 5 mg./l.) of 7:12-dihydroxy-3-keto- and 3:12-dihydroxy-7-keto-cholanic acids; the form in which these acids occur in the bile was not determined.

2. These keto acids may arise by bacterial action in the intestine, followed by absorption and excretion of the products. Bacteria other than *Alcaligenes faecalis* are probably concerned.

3. A futher statement is made about the Hammarsten test for cholic acid derivatives.

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