Steroids. Part XI. Isolation of Cholegenin and* iso*Cholegenin from Ox-bile.*

By N. J. Antia, Y. Mazur, R. R. Wilson, and F. S. Spring.

[Reprint Order No. 4881.]

The neutral fraction from ox-bile is shown to contain, in addition to much cholesterol, pregnane-3α : 20β-diol and two dihydric alcohols, $C_{27}H_{44}O_4$, named cholegenin and *iso*cholegenin. Cholegenin is converted into *iso*-cholegenin by the action of mineral acid.

It has frequently been reported that symptoms are considerably alleviated in patients suffering from rheumatoid arthritis if jaundice intervenes (for bibliography see Hench, Kendall, Slocumb, and Polley, *Assoc. Staff Meet., Mayo Clinic*, 1949, **24**, 181). This prompted us to examine the neutral fraction of ox-bile with the initial object of identifying the neutral steroid components. Apart from metabolic products intermediate between cholesterol and bile acids, there was reason to believe that bile contains pregnane derivatives which may be of value in a study of the relation between steroid metabolism and arthritis.

Pearlman (*J. Amer. Chem. Soc.*, 1944, **66**, 806) in an exploratory examination of the neutral, non-saponifiable fraction of ox-bile, isolated, in addition to cholesterol, five compounds, A—E, one of which, compound B, was identified later as *allo*pregnane-3β : 20β-diol (Pearlman, *J. Biol. Chem.*, 1946, **166**, 473) : in a subsequent examination of ox-bile, he failed to isolate this pregnanediol. Pearlman and Cerceo later (*ibid.*, 1948, **176**, 847) isolated 3β-hydroxypregnan-20-one, pregnane-3α : 20β-diol, and *ætio*cholane-3α : 17β-diol from the neutral fraction from the bile of pregnant cows.

The procedure adopted by us for the isolation of the neutral fraction from ox-bile differs considerably from that used by Pearlman. After removal of the acids by treatment of the bile with cold barium hydroxide solution followed by digestion of the mass

* Part X, *J.*, 1954, 328.

with chloroform, the neutral fraction was extracted with ether. The ether-soluble material was then extracted with light petroleum, and the insoluble fraction acetylated and chromatographed on alumina. In addition to a considerable amount of cholesteryl acetate, small quantities of the di- and mono-acetates of a compound, $C_{27}H_{44}O_4$, were isolated. This compound is a steroid sapogenin (see following paper) and, since it has been isolated from all samples of bile examined by us and is, we believe, an invariable constituent of ox-bile, we name it cholegenin. The mono- and di-acetates were interrelated by hydrolysis of each to cholegenin, and by further acetylation of the mono- to the di-acetate. Cholegenin and acetic anhydride in pyridine give the diacetate, which is partially hydrolysed on alumina to the monoacetate. The fraction soluble in light petroleum consisted essentially of cholesterol.

In an alternative method, the ether-soluble neutral fraction from bile was hydrolysed by alcoholic potassium hydroxide, and the non-saponifiable matter extracted with cold light petroleum which removed a fraction containing much cholesterol. The insoluble fraction was next extracted with boiling light petroleum, and the insoluble material chromatographed on alumina. Removal of a considerable amount of cholesterol from the fraction extracted with hot light petroleum was followed by chromatography of the residue on alumina. In this way, three homogeneous compounds were isolated, *viz.*, cholegenin, an isomer which we name *iso*cholegenin, and pregnane-3α : 20β-diol.

Pregnane-3α : 20β-diol was identified by direct comparison with a specimen prepared from progesterone,* hydrogenation of which over palladised charcoal yielded a mixture of pregnane- and *allo*pregnane-3 : 20-dione separated by trituration with methanol. Reduction of pregnane-3 : 20-dione with lithium aluminium hydride, followed by acetylation, yielded pregnane-3α : 20β-diol diacetate identical with the specimen isolated from bile. This identification was confirmed by a comparison of the corresponding diols. It is probable that Pearlman's compound D is pregnane-3α : 20β-diol; compound D was not obtained by Pearlman in sufficient quantity for analysis or for the determination of its specific rotation; the constants of its acetyl derivative (formulated as $C_{26}H_{42}O_4$ by Pearlman), m. p. 111°, $[\alpha]_D$ +72° (in EtOH), closely simulate those of pregnane-3α : 20β-diol diacetate {$C_{25}H_{40}O_4$, m. p. 113°, $[\alpha]_D$ +69° (in EtOH)}.

*iso*Cholegenin, $C_{27}H_{44}O_4$, is probably identical with Pearlman's compound C. Pearlman formulated compound C, which is not precipitated by digitonin, as $C_{25-26}H_{40-42}O_4$ and characterised it by the formation of a monoacetate under mild acetylation conditions. *iso*Cholegenin also is not precipitated by digitonin and on mild acetylation gives a monoacetate, with a smaller quantity of a diacetate. More drastic acetylation yields the diacetate; alkaline hydrolysis of the mono- or the di-acetate yields *iso*cholegenin. *iso*Cholegenin is probably formed from cholegenin during the isolation: it is obtained from the latter by the action of mineral acids. Cholegenin is the major component of the cholesterol-free neutral fraction from ox-bile, and Pearlman's failure to isolate it may well be due to its conversion into *iso*cholegenin (compound C) during the isolation procedure used by him.

Experimental

Specific rotations were measured in chloroform solution (unless otherwise stated) in a 1-dm. tube at approx. 15°. Grade II/III alumina, and a light petroleum fraction of b. p. 40—60° were used for chromatography.

Isolation of Cholegenin Diacetate and Cholegenin Monoacetate from Ox-bile.—Approx. 40 gallons of ox-bile were stirred with a solution of barium hydroxide [5 kg. $Ba(OH)_2,8H_2O$] in water (31 l.), and the mixture was extracted with chloroform (125 l.). The chloroform extract was filtered and the solvent evaporated under reduced pressure. The viscous dark green residue was dried in a vacuum over phosphoric oxide for 2 days, powdered, and extracted with ether (Soxhlet). Concentration of the extract gave a light brown powder (148 g., dried in a vacuum over P_2O_5). The powder was repeatedly extracted with boiling light petroleum (6 × 500 c.c.). The insoluble solid (10 g.) was separated; the combined, filtered extracts were

* The progesterone was given to us by Dr. C. L. Hewett, Organon Laboratories, Newhouse, Lanarkshire, to whom we express our best thanks.

concentrated, and the separating solid was collected and recrystallised from methanol, to yield cholesterol, m. p. and mixed m. p. 148—149°, $[\alpha]_D$ −40° (*c*, 3·5). Concentration of the light petroleum extracts gave additional amounts of cholesterol (total, 73 g.).

A sample (5 g.) of the solid insoluble in boiling light petroleum was kept with acetic anhydride (5 c.c.), pyridine (5 c.c.) and benzene (20 c.c.) at room temperature for 24 hr. and then heated under reflux for 2 hr. A solution of the acetylated product, isolated in the usual manner, in light petroleum was chromatographed on a column (27 × 2·5 cm.) of alumina. The column was washed with light petroleum (1550 c.c.) and then with light petroleum–benzene (19 : 1, 400 c.c.; 9 : 1, 600 c.c.; 4 : 1, 500 c.c.) which eluted a fraction (0·92 g.; m. p. 100—110°), recrystallisation of which from methanol yielded cholesteryl acetate as needles, m. p. and mixed m. p. 115—116°. Continued elution with light petroleum–benzene (1 : 1, 500 c.c.; 1 : 4, 450 c.c.) and benzene (300 c.c.) gave a fraction (0·59 g.; m. p. 140—170°), four crystallisations of which from ether–methanol gave *cholegenin diacetate* as fibrous needles, m. p. 174—175°, $[\alpha]_D$ −21°, −19° (*c*, 0·6, 0·7) (Found: C, 72·4, 72·25; H, 9·5, 9·65. $C_{31}H_{48}O_6$ requires C, 72·1; H, 9·4%). Continued elution with benzene (350 c.c.) and benzene–ether (4 : 1; 300 c.c.) gave a fraction (0·36 g.) which did not crystallise. A fourth fraction (0·98 g.; m. p. 130—180°) obtained by washing the column with benzene–ether (4 : 1, 600 c.c.; 1 : 1, 600 c.c.) and ether (500 c.c.) was crystallised four times from ether–light petroleum (b. p. 60—80°), to yield *cholegenin monoacetate* as needles, m. p. 192—193°, $[\alpha]_D$ −8°, −7° (*c*, 0·8) (Found: C, 73·5; H, 10·1. $C_{29}H_{46}O_5$ requires C, 73·4; H, 9·8%).

Cholegenin.—A solution of cholegenin diacetate (135 mg.) in methanolic potassium hydroxide (3%, 200 c.c.) was heated under reflux for 2 hr. The product was isolated by means of ether and crystallised from ether–light petroleum (b. p. 60—80°), to yield *cholegenin* as needles, m. p. 193°, $[\alpha]_D$ −27°, −25° (*c*, 0·8, 0·75) (Found: C, 75·2, 74·8; H, 10·4, 10·6. $C_{27}H_{44}O_4$ requires C, 74·95; H, 10·25%). Cholegenin does not give a colour with tetranitromethane in chloroform, does not show selective absorption between 2000 and 3500 Å, and does not give a precipitate with aqueous-alcoholic digitonin.

When cholegenin monoacetate (50 mg.) was similarly hydrolysed and the product crystallised from ether–light petroleum (b. p. 60—80°), it yielded cholegenin as needles, m. p. and mixed m. p. 193°, $[\alpha]_D$ −25° (*c*, 1·0).

Cholegenin (30 mg.) was kept in benzene (5 c.c.) with acetic anhydride (1 c.c.) and pyridine (1 c.c.) at room temperature for 24 hr. The product, isolated by means of ether and crystallised from ether–methanol, gave cholegenin diacetate as needles, m. p. and mixed m. p. 174—175°, $[\alpha]_D$ −19° (*c*, 0·6).

Cholegenin monoacetate (20 mg.) with pyridine (2 c.c.) and acetic anhydride (2 c.c.) at room temperature gave cholegenin diacetate, needles [from ether–light petroleum (b. p. 60—80°)], m. p. and mixed m. p. 173°.

Cholegenin diacetate (400 mg.) was adsorbed on alumina (10 g.) from a benzene solution, and the moist column kept for 7 days. Benzene (55 c.c.) then eluted a crystalline fraction (106 mg.; m. p. 170°) which after recrystallisation from ether–methanol gave the diacetate as needles, m. p. and mixed m. p. 173—174°. Continued washing of the column with benzene (125 c.c.) and benzene–ether (1 : 4; 50 c.c.) gave a fraction (55 mg.) which after recrystallisation from ether–light petroleum (b. p. 60—80°) gave cholegenin monoacetate as needles, m. p. and mixed m. p. 193°. A third fraction (20 mg.; m. p. 185—190°) was eluted with benzene–ether (1 : 1; 100 c.c.) and after recrystallisation from ether–light petroleum (b. p. 60—80°) gave cholegenin as needles, m. p. and mixed m. p. 193°.

Isolation of Cholegenin, Pregnane-3α : 20β-diol, and iso*Cholegenin.*—The dried solid (158 g.) obtained as described above from 40 gallons of ox-bile by treatment with barium hydroxide followed by extraction with chloroform was heated under reflux with ether for 24 hr. The ether-insoluble fraction (10 g.) was discarded. The solid obtained after removal of the solvent from the dried solution was heated under reflux with methanolic potassium hydroxide (5%; 3 l.). The non-saponifiable fraction (130 g.), isolated by means of ether, was heated under reflux for 15 min. with light petroleum (500 c.c., b. p. 60—80°), and the mixture cooled to room temperature and kept overnight. The insoluble solid (36 g.) was collected and heated under reflux with light petroleum (2 l.) for 36 hr. Filtration of the hot mixture gave a filtrate and a solid A (8·3 g.; see p. 1922). Removal of solvent from the filtrate gave a solid (27·7 g.) which readily crystallised from acetone, to yield cholesterol (21 g.), m. p. and mixed m. p. 148—149°. Crystallisation of the residue obtained by evaporation of the acetone mother-liquor, from methanol, gave more cholesterol (4·7 g.). Evaporation of the methanol mother-liquors provided an amorphous solid (1·98 g.), a solution of which in benzene (20 c.c.) was chromato-

graphed on alumina (50 g.). Benzene–ether (3 : 1, 600 c.c.) eluted a solid (903 mg.; m. p. 135—142°), crystallisation of which from methanol yielded cholesterol (750 mg.), m. p. and mixed m. p. 148—149°. Continued elution with benzene–ether (3 : 1, 300 c.c.) eluted a second fraction (236 mg.; m. p. 142—220°) which was triturated with a small volume of ether. The ether-soluble fraction crystallised from methanol, to yield cholesterol (149 mg.), m. p. and mixed m. p. 147°. A third fraction (243 mg.; m. p. 216—240°) obtained by washing the column with benzene–ether (3 : 1, 600 c.c.) was combined with the ether-insoluble part of the second fraction and, crystallised from ethyl acetate, gave needles (105 mg.), m. p. 218—219° (fraction 3*a*, see below.

Cholegenin. Continued washing of the column with benzene–ether (3 : 1, 150 c.c.; 1 : 1, 900 c.c.; 1 : 3, 450 c.c.) eluted a fourth fraction (314 mg.; m. p. 175—180°) which was thrice crystallised from ether–light petroleum (b. p. 60—80°), to yield cholegenin as needles, m. p. and mixed m. p. 193°, $[\alpha]_D$ −25° (*c*, 0·9).

Pregnane-3α : 20β-diol diacetate. (*a*) Fraction 3*a* was acetylated with pyridine and acetic anhydride, and the product was isolated by means of ether. A solution of this in light petroleum (10 c.c.) was filtered through neutral alumina (18 g.). A fraction (21 mg.; gum) eluted by light petroleum (175 c.c.) and light petroleum–benzene (3 : 1, 75 c.c.; 1 : 1, 75 c.c.) was not examined. Continued elution with light petroleum–benzene (1 : 1; 75 c.c.) gave a fraction (26 mg.; m. p. 110—111°) which on crystallisation from methanol followed by sublimation in a vacuum at 70—80° yielded pregnane-3α : 20β-diol diacetate (20 mg.) as needles, m. p. 113°, $[\alpha]_D$ +66° (*c*, 0·8) (Found : C, 74·1; H, 9·9. Calc. for $C_{25}H_{40}O_4$: C, 74·2; H, 10·0%).

(*b*) Hydrogenation of progesterone (1 g.) in ethanol (100 c.c.) in presence of palladised charcoal (10%; 80 mg.) (cf. Butenandt and Fleischer, *Ber.*, 1935, **68**, 2094) and trituration of the product with methanol (50 c.c.) gave *allo*pregnane-3 : 20-dione (insoluble); material recovered from the filtrate, when crystallised thrice from ether–light petroleum (b. p. 60—80°), yielded crude pregnane-3 : 20-dione as needles, m. p. 115°. A solution of the diketone in tetrahydrofuran (50 c.c.) was heated with lithium aluminium hydride (0·15 g.) under reflux for 30 min. The product, isolated in the usual manner, was triturated with cold ether (20 c.c.), and the insoluble fraction twice crystallised from chloroform–ethyl acetate, to yield pregnane-3α : 20β-diol, needles, m. p. 236°. Acetylation of the diol (210 mg.) with pyridine and acetic anhydride gave the diacetate, needles (from methanol), m. p. 113°, $[\alpha]_D$ +65°, +69° (*c*, 1·3, 1·2 in EtOH) (cf. Sarett, *J. Amer. Chem. Soc.*, 1949, **71**, 1179; Pearlman and Cerceo, *loc. cit.*; Meystre and Miescher, *Helv. Chim. Acta*, 1946, **29**, 33). A mixture with the specimen described under (*a*) had m. p. 113°.

Hydrolysis of pregnane-3α : 20β-diol diacetate (15 mg.) from ox-bile for 2 hr. with hot 3% methanolic potassium hydroxide (50 c.c.), followed by crystallisation (twice) from chloroform–ethyl acetate, yielded pregnane-3α : 20β-diol as fine needles, m. p. 235—236°, undepressed in m. p. when mixed with the specimen prepared from progesterone. Pearlman (1944, *loc. cit.*) gives m. p. 232—233° for compound D.

iso*Cholegenin.* (*a*) After removal of pregnane-3α : 20β-diol diacetate from the fraction 3*a* chromatogram, continued washing of the column with light petroleum–benzene (1 : 1; 225 c.c.) eluted a gum (3 mg.), whereafter benzene (75 c.c.) eluted a fraction (8 mg.; m. p. 226—228°) crystallisation of which from ether–methanol yielded iso*cholegenin diacetate* as long needles, m. p. 232—233°, $[\alpha]_D$ −38° (*c*, 0·8) (Found : C, 71·9; H, 9·6. $C_{31}H_{48}O_6$ requires C, 72·1; H, 9·4%). A final fraction (50 mg.; m. p. 183—184°) was obtained from the chromatogram by continued washing with benzene (300 c.c.) and then with benzene–ether (9 : 1; 150 c.c.). Four crystallisations of this from ether–light petroleum (b. p. 60—80°) yielded iso*cholegenin monoacetate* (10 mg.) as prisms, m. p. 187°, $[\alpha]_D$ −42° (*c*, 0·9) (Found : C, 72·9; H, 10·1. $C_{29}H_{46}O_5$ requires C, 73·4; H, 9·8%). Pearlman (1944, *loc. cit.*) gives m. p. 187° for the acetate of compound C.

A solution of *iso*cholegenin monoacetate (10 mg.) in 3% methanolic potassium hydroxide (50 c.c.) was heated under reflux for 2 hr. *iso*Cholegenin, isolated by means of ether, separated from ethyl acetate as needles which, after sublimation in a high vacuum at 200°, had m. p. 256—257° (decomp.), $[\alpha]_D$ −65°, −67° (*c*, 0·5, 0·6) (Found : C, 75·0, 74·8; H, 10·5, 10·6. $C_{27}H_{44}O_4$ requires C, 74·95; H, 10·25%). Pearlman (1944, *loc. cit.*) gives m. p. 260° for compound C. *iso*Cholegenin does not give a colour with tetranitromethane in chloroform, does not show selective absorption between 2000 and 3500 Å, and does not give a precipitate with aqueous-alcoholic digitonin.

*iso*Cholegenin (181 mg.) was acetylated with pyridine and acetic anhydride at room temperature, then chromatographed in light petroleum (10 c.c.) on alumina (8 g.). Light petroleum–benzene (3 : 2; 150 c.c.) eluted a solid (20 mg.; m. p. 217—230°), crystallisation of

which from ether–methanol gave *iso*cholegenin diacetate (9 mg.) as needles, m. p. and mixed m. p. 232—233°. Continued washing of the column with light petroleum–benzene (1 : 1; 200 c.c.) gave a fraction (55 mg.; m. p. 172—183°), which, after two crystallisations from ether–light petroleum (b. p. 60—80°), yielded *iso*cholegenin monoacetate as prisms, m. p. and mixed m. p. 187°.

*iso*Cholegenin (70 mg.) was refluxed with acetic anhydride (20 c.c.) for 14 hr. Recrystallisation of the product from ether–methanol yielded *iso*cholegenin diacetate as long needles (55 mg.), m. p. and mixed m. p. 232°.

(*b*) Cholegenin (200 mg.) in ethanol (50 c.c.) was refluxed with concentrated hydrochloric acid (10 c.c.) for 5 hr. The product was isolated by means of chloroform and crystallised several times from chloroform–ethyl acetate, to give *iso*cholegenin as fine needles, m. p. 252° (decomp.), $[\alpha]_D$ −67° (*c*, 0·8); a mixture with *iso*cholegenin described above was undepressed in m. p.

Dry hydrogen chloride was passed for 3 hr. through a solution of cholegenin (60 mg.) in chloroform (50 c.c.). After the mixture had been kept for 18 hr. at room temperature, the product was isolated by means of chloroform, to give a crystalline product, m. p. 214—220°, which after three recrystallisation from chloroform–ethyl acetate gave *iso*cholegenin as needles, m. p. and mixed m. p. 248—252°.

Examination of Solid A.—A solution of solid A (8·3 g.) in benzene (100 c.c.) was filtered through alumina (250 g.), and the column was washed with light petroleum–benzene (1 : 2, 200 c.c.), giving a crystalline fraction (6·0 g.; m. p. 144—147°), recrystallisation of which from methanol yielded cholesterol as plates, m. p. and mixed m. p. 147—148°. Continued washing with the same solvent mixture (500 c.c.) gave a second fraction, which was crystallised from chloroform–ethyl acetate, to give *iso*cholegenin as needles (181 mg.), m. p. and mixed m. p. 248—252° (decomp.).

The third fraction (1·3 g.; m. p. 170—180°) eluted with ether (375 c.c.) was acetylated by pyridine and acetic anhydride, the product being isolated by means of ether. A solution of this in light petroleum (50 c.c.) was filtered through neutral alumina (30 g.). The column was washed with light petroleum (675 c.c.), and with light petroleum–benzene (9 : 1, 300 c.c.; 4 : 1, 225 c.c.), to give a crystalline fraction (0·95 g.; m. p. 165—173°), recrystallisation of which from ether–methanol yielded cholegenin diacetate, m. p. and mixed m. p. 173—174°, $[\alpha]_D$ −23°, −21° (*c*, 1·1, 1·4). The column was then washed with light petroleum–benzene (4 : 1, 375 c.c.; 2 : 1, 75 c.c.; 1 : 1, 150 c.c.) which eluted a fraction (193 mg.; m. p. 138—168°). A third fraction (140 mg.; m. p. 183—190°) was obtained by elution with benzene (75 c.c.) and benzene–ether (9 : 1, 150 c.c.). Crystallisation of this fraction from ether–light petroleum (b. p. 60—80°) gave cholegenin monoacetate as needles, m. p. and mixed m. p. 192—193°, $[\alpha]_D$ −7° (*c*, 0·8).

The second fraction was hydrolysed for 2 hr. in hot ethanolic potassium hydroxide (3%; 50 c.c.). The product was isolated by means of ether (190 mg.), disssolved in benzene (20 c.c.), and filtered through alumina (8 g.). Benzene–ether (9 : 1, 525 c.c.; 1 : 1, 150 c.c.) and ether (150 c.c.) eluted a solid (180 mg.; m. p. 187—188°) which, after crystallisation from ether–light petroleum (b. p. 60—80°), gave cholegenin, m. p. and mixed m. p. 193°.

Grateful acknowledgment is made of a grant in aid from the Nuffield Foundation (Oliver Bird Fund). We also express our indebtedness to Messrs. T. & H. Smith, Ltd., Edinburgh, who undertook large-scale preparations of the ox-bile neutral fraction.

THE ROYAL TECHNICAL COLLEGE, GLASGOW. [*Received, December 11th,* 1953.]