hydrolysis, Freudenberg and Harder[5] method), 10.52 cc.

Summary

1. There is reported the synthesis, in crystalline form, of the first acyclic sugar orthoacetate (IV), designated 1-thioethoxy-*aldehydo*-D-galactose ethyl 1,2-orthoacetate tetraacetate.

(5) K. Freudenberg and M. Harder, *Ann.*, **433**, 230 (1923).

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[CONTRIBUTION FROM THE PHYSIOLOGICAL LABORATORIES, CLARK UNIVERSITY]

A Study of the Neutral, Non-Saponifiable Fraction of Ox Bile[1]

BY W. H. PEARLMAN[1a]

A theory[2] postulating a biological conversion of cholesterol to the bile acids has recently received experimental support in an investigation[3] carried out with the aid of deuterium. One might expect to find metabolic products intermediate between cholesterol and the bile acids in bile since it contains these steroids in large amount. Studies of the comparative biochemistry of the bile have already furnished clues as to the nature of the hypothetical intermediates. The bile of elasmobranchs[2] contains a polyhydric alcohol, scymnol ($C_{27}H_{46}O_5$) which is related to cholesterol; it can be readily degraded in the laboratory to cholic acid.[4,5] Other alcohols of an alleged steroid nature have been isolated from the bile of frogs[6] and toads[7,8]; of these, pentahydroxybufostane ($C_{28}H_{50}O_5$) can be oxidized *in vitro* to cholic acid.[7] A mechanism for the biological oxidation of the side-chain of steroids with 27 and 28 carbon atoms has been suggested by Kazuno.[7]

An exploratory examination of the neutral fraction of ox bile failed to reveal the presence of scymnol, etc. On the other hand, five compounds (other than cholesterol[9]) were isolated in small amount. Although the empirical formulas (see Table I) point to a steroid structure, these compounds could not be identified with any known steroids. Structural proof must wait until more material is available.

Compound B, m. p. 192–193°, has an empirical formula identical with that of pregnandiol. Since Compound B is digitonin-precipitable, pregnandiol-3(β),20(α) was suspected to be the isomer in question. This steroid had been prepared by Butenandt and Müller[14] and they reported a melting point of 189–190.5° uncor. Marker, *et al.*,[15] had previously prepared the isomer from pregnandiol but their preparation melted at 182°. This discrepancy in melting points was noted by Butenandt and Müller; they raised a doubt regarding the nature of their own preparation, stating that isomerization of the C-17 side-chain might have occurred since the reduction of the 20-keto group of the intermediary steroid had been carried out in an alkaline medium. The writer prepared pregnandiol-3(β),20(α), following the procedure of Marker, *et al.*,[15] in all respects except that the intermediate 20-acetate of pregnandiol-3(α),20(α), was isolated. The final product melted at 182°; purification by chromatographic analysis did not raise the melting point. The dibenzoate (not previously described) melts at 167–168°. Since the dibenzoate of Compound B melts at 234–235°, it is clear that Compound B cannot be identical with pregnandiol-3(β),20(α). Compound B thus remains unidentified for the time being; it is probably an (*allo*) pregnane derivative containing a 3(β)-hydroxyl group.

TABLE I

COMPOUNDS (OTHER THAN CHOLESTEROL) ISOLATED FROM THE NEUTRAL, NON-SAPONIFIABLE FRACTION OF OX BILE

Compd.	M. p.,[12] °C.	Tentative empirical formula	Derivatives: Type	Derivatives: M. p.,[12] °C.
A	300	$C_{27}H_{40}O_3$	Monoacetate	216–217
			Monobenzoate	155–157
B	192–193	$C_{21}H_{36}O_2$	Dibenzoate	234–235
C	255–257	$C_{25-26}H_{40-42}O_4$	Monoacetate	187
D	232–233	$C_{24}H_{40}O_3$[13]	Monoacetate	111
E	202	$C_{24}H_{42}O_4$	Diacetate	142.5

Compound C contains either 25 or 26 carbon atoms. This is an unusual number for naturally occurring steroids but it might be mentioned that a C-26 acid has been obtained from bile.[6]

The ever-recurrent question of artifact formation deserves mention in this report but, since the

(1) Aided by a grant from G. D. Searle and Company.

(1a) Present Address: Department of Biology, Princeton University, Princeton, N. J.

(2) L. F. Fieser, "The Chemistry of Natural Compounds Related to Phenanthrene," Reinhold Publishing Corp., New York, N. Y., 1937.

(3) K. Bloch, B. N. Berg and D. Rittenberg, *J. Biol. Chem.*, **149**, 511 (1943).

(4) H. Asikari, *J. Biochem.* (Japan), **29**, 319 (1939).

(5) W. Bergmann and Wm. T. Pace, THIS JOURNAL, **65**, 477 (1943).

(6) Y. Kurauti and T. Kazuno, *Z. physiol. Chem.*, **262**, 53 (1939).

(7) T. Kazuno, *ibid.*, **266**, 11 (1940).

(8) H. Makino, *ibid.*, **220**, 49 (1933).

(9) No attempt was made to isolate dihydrocholesterol which Schoenheimer, *et al.*,[10] have shown to be present to the extent of 1–2% in cholesterol from various organs; Pertzborn[11] reports 3.6% dihydrocholesterol in ox bile.

(10) R. Schoenheimer, H. V. Behring and R. Hummel, *ibid.*, **192**, 93 (1930).

(11) R. Pertzborn, *Z. ges. exptl. Med.*, **101**, 350 (1937).

(12) All m. p.'s in this paper are corrected.

(13) Empirical formula deduced from analytical data on acetate.

(14) A. Butenandt and G. Müller, *Ber.*, **71**, 191 (1938).

(15) R. E. Marker, O. Kamm, E. L. Wittle, T. S. Oakwood, E. J. Lawson and J. F. Laucius, THIS JOURNAL, **59**, 2291 (1937).

steroid structure of Compounds A-E is not yet established, any discussion as to the possible origin of these from either cholesterol or the bile acids during the course of isolation would be altogether premature.

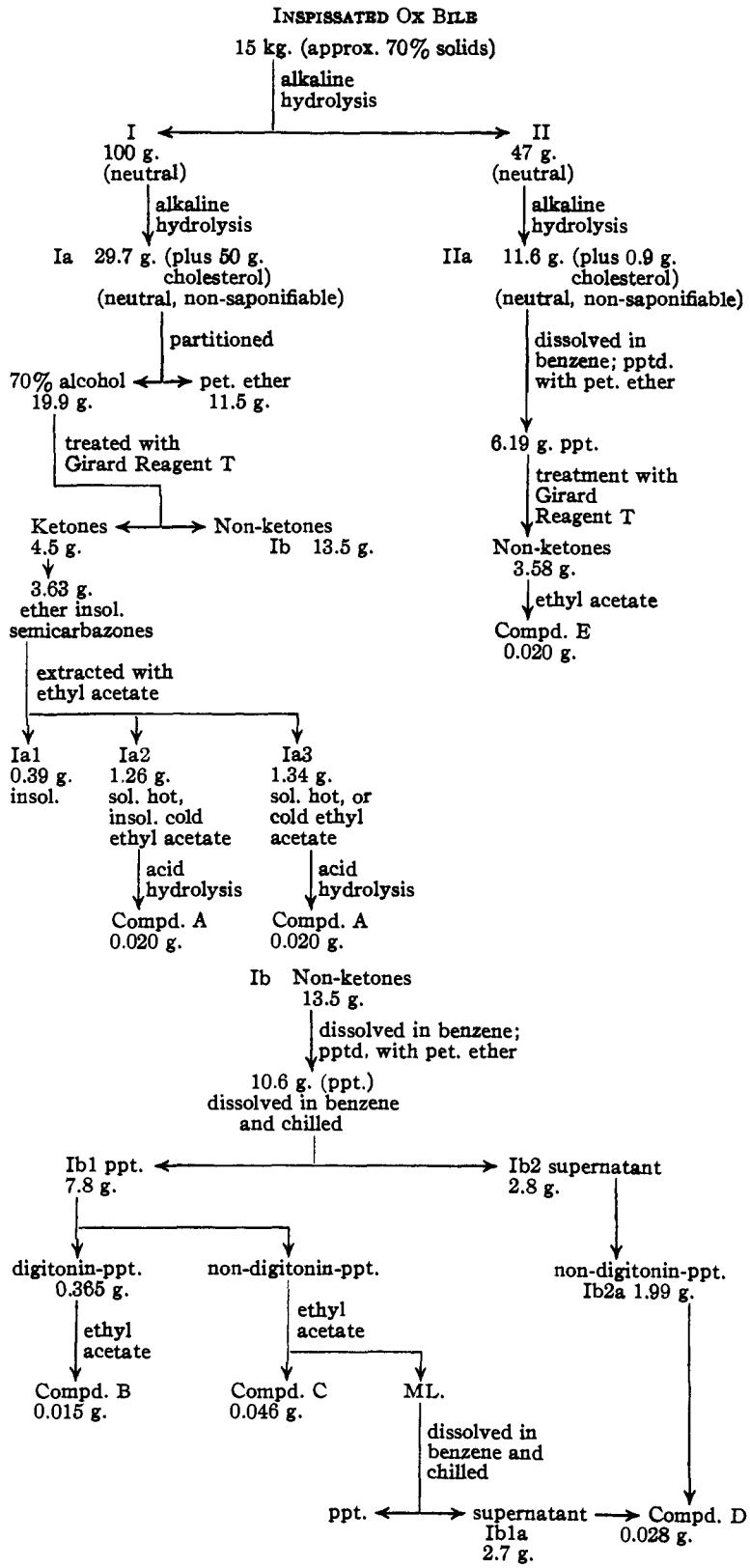
This investigation was undertaken at the kind invitation of Professor Gregory Pincus. The author is grateful to Miss Mary Ruth Jones for technical assistance.

Experimental

The Non-saponifiable Fraction of Ox Bile.—Fifteen kilograms of inspissated ox bile, containing approximately 70% solids, was hydrolyzed for eighteen to twenty hours at 104° in 27% solution with 6% sodium hydroxide. Addition of hydrochloric acid to the hydrolyzate gave a precipitate of crude bile acids which was extracted with ethyl acetate. The extract was evaporated, the residue dissolved in dilute alkali and extracted with ether. The ether extract on evaporation weighed 100 g. (Fraction I). The crude bile acids were crystallized from alcohol. The mother liquor was evaporated and the residue crystallized from 55% acetic acid. The mother liquor was evaporated, the residue dissolved in dilute alkali, and extracted with ether. The ether extract on evaporation weighed 47 g. (Fraction II).[16]

Fractions I and II still contained considerable amounts of saponifiable material. These fractions were therefore dissolved in 90% methanol containing 5% potassium hydroxide and refluxed for one hour. The hydrolyzate of Fraction I crystallized on cooling to room temperature, yielding 50 g. of crude cholesterol; an additional 29.7 g. of neutral material (Fraction Ia) was obtained. Fraction II likewise yielded 11.6 g. of neutral material (Fraction IIa) in addition to 0.9 g. of crude cholesterol. A very much abbreviated description of the fractionation of the neutral, non-saponifiable material follows (see flow sheet).

Compound A.—Fraction Ia (29.7 g.) was partitioned between 70% alcohol and low boiling petroleum ether (30–60°). The 70% alcohol fraction (19.9 g.) was treated with Girard reagent T[19] to give 4.5 g. of ketones, from which was obtained 3.63 g. of ether-insoluble semicarbazones. Treatment of the latter with boiling ethyl acetate yielded three fractions. Fraction Ia1 (0.39 g.) was insoluble in hot or cold ethyl acetate. Fraction Ia2 (1.26 g.) was soluble in hot ethyl acetate but insoluble in the cold and Fraction Ia3 (1.34 g.) was soluble in hot or cold ethyl acetate. These fractions were hydrolyzed with acid under mild conditions. The hydrolyzate of Fraction Ia1 failed to yield any crystalline products. The hydrolyzate of Fraction Ia2, after the re-



(16) The processing was carried out in the laboratories of G. D. Searle and Company; the author is especially indebted to Dr. R. T. Dillon.

moval of a small amount of digitonin-precipitable material, yielded, on treatment with cold methanol, a small amount of crystalline material. Repeated crystallization from ethyl acetate gave 8.5 mg. of compact prisms, m. p. 300°. Compound A develops a cherry-red color when dissolved in concd. sulfuric acid; the test is very sensitive.

Anal. Calcd. for $C_{27}H_{40}O_3$: C, 78.60; H, 9.78; mol. wt., 413. Found: C, 78.64; H, 9.86; mol. wt. (Rast), 395.

The mother liquors yielded an additional 11 mg., m. p. 284–286°. It was dissolved in 1 cc. of dry pyridine and 0.5 cc. of acetic anhydride was added. After standing at room temperature for twenty-four hours, the reaction mixture was poured into water, the precipitate was filtered and washed well with water. It was once recrystallized from ethanol to give 5 mg. of needles, m. p. 216–217°. The derivative did not exhibit any absorption bands in the ultraviolet spectrum.

Anal. Calcd. for $C_{29}H_{42}O_4$, monoacetate of Compound A: C, 76.62; H, 9.31. Found: C, 76.83; H, 9.30.

The hydrolyzate of Fraction Ia3, after the removal of a small amount of digitonin-precipitable material, was dissolved in 250 cc. of carbon tetrachloride and chromatographed on 12 g. of aluminum oxide (Merck, standardized according to Brockmann).

TABLE II

No. of fraction	Eluent	Vol., cc.	Eluate, mg.	Crystallized once from ethyl acetate Mg.	M. p., °C.
1	CCl_4	300	344		
2	CCl_4	130	111	8	
3	CCl_4 (1% ethanol)	200	462	33	277–280
4	Ethanol	35	141		

Fraction 3 on recrystallization from ethyl acetate gave 20 mg. of compact prisms, m. p. 290–293° (Compound A). Ten mg. was dissolved in 1 cc. of dry pyridine and 0.2 cc. of benzoyl chloride added. After standing at room temperature for twenty-four hours, the reaction mixture was decomposed in dilute sodium carbonate solution. The precipitate was filtered, washed well with water and dried; it was extracted with a little petroleum ether and the insoluble residue crystallized from methanol. Repeated crystallization from methanol gave 3.5 mg. of fine needles, m. p. 155–157°. The ultraviolet absorption curve of the benzoate derivative of Compound A exhibited maxima at 2310 A. (ϵ = 13,470) and at 2720 Å. (ϵ = 973). The presence of only one benzoyl group is indicated if comparison is made with the ultraviolet absorption curve of the dibenzoate of 7(β)-hydroxycholesterol (see below).

Compound B.—Fraction Ibl (7.8 g.) was treated with an excess of a 2% digitonin solution in 70% methanol. The precipitate was filtered, washed with a little 70% methanol and thoroughly desiccated. After extraction with dry ether, the digitonides were worked up by dissolving in pyridine and adding an excess of dry ether. The digitonin-precipitable material weighed 365 mg.; on treatment with ethyl acetate, it yielded 56 mg. of crystalline product. Repeated crystallization from this solvent gave 7 mg., m. p. 192–193°.

Anal. Calcd. for $C_{21}H_{36}O_2$: C, 78.69; H, 11.33. Found: C, 78.65; H, 11.30.

An additional 8 mg., m. p. 187–189°, was obtained from the mother liquors. The benzoylated product crystallized in beautiful needles from alcohol, yielding 8 mg., m. p. 234–235°.

Anal. Calcd. for $C_{35}H_{44}O_4$, dibenzoate of Compound B: C, 79.50; H, 8.40. Found: C, 79.25; H, 8.46.

The ultraviolet absorption curve of the dibenzoate of Compound B exhibited maxima at 2310 A. (ϵ = 27,700) and at 2720 Å. (ϵ = 1985). The presence of two benzoyl groups is thus indicated if comparison is made with the ultraviolet absorption curve of the dibenzoate of 7(β)-hydroxycholesterol[17] [maxima at 2310 Å. (ϵ = 30,000) and at 2720 Å. (ϵ = 1857)].

Pregnandiol-3(β),20(α) which had been prepared by the method of Marker, *et al.*,[15] was adsorbed on aluminum oxide and eluted with carbon tetrachloride containing increasing amounts of ethanol. The m. p. of the preparation was not raised above 182°. The product was benzoylated in the usual manner. It crystallized from alcohol as plates, m. p. 167–168°.

Anal. Calcd. for $C_{35}H_{44}O_4$: C, 79.50; H, 8.40. Found: C, 79.43; H, 8.30.

Compound C.—Fraction Ibl (7.8 g.), after the removal of the digitonin-precipitable material, was treated with ethyl acetate to yield 65 mg. of a crystalline product, m. p. 230–233°. It was crystallized repeatedly from ethyl acetate to give 15 mg. of long white needles, m. p. 255–257°.

Anal. Calcd. for $C_{35}H_{40}O_4$: C, 74.21; H, 9.96; mol. wt., 405. Calcd. for $C_{26}H_{42}O_4$: C, 74.61; H, 10.11; mol. wt., 419. Found: C, 74.47; H, 10.18; mol. wt. (Rast), 386.

The mother liquors of Compound C were worked up to yield a total of 151 mg. of crude crystalline material. It was dissolved in 5 cc. of pyridine and 2.5 cc. of acetic anhydride added. After standing at room temperature for twenty-four hours, the reaction mixture was poured into water and extracted with ether. The ether extract was washed with dilute hydrochloric acid, dilute sodium carbonate and finally with water; evaporation of the extract yielded 102 mg. of a colorless glassy product. It was dissolved in 25 cc. of a pentane:benzene (4:1) mixture and chromatographed on 7 g. of aluminum oxide.

TABLE III

No. of fraction	Eluent	Eluate, mg.	Crystallized once from methanol Mg.	M. p., °C.
1	Pentane:benzene (4:1) 90 cc.	2.7 (oil)		
2	Pentane:benzene (1:1) 40 cc.	25.5 (oil)	5.6	225–228
3	Pentane:benzene (1:1) 20 cc.	14.9 (oil)	5.9	176–178
4	Pentane:benzene (1:1) 50 cc.	23.8 (cryst.)	9.5	185–186
5	Benzene 95 cc.	38.2 (cryst.)	33.2	181–183

Fractions 4 and 5 were repeatedly crystallized from methanol to yield, in all, 31 mg. of needles, m. p. 187°.

Anal. Calcd. for $C_{27}H_{42}O_5$, monoacetate of $C_{25}H_{40}O_4$: C, 72.62; H, 9.47. Calcd. for $C_{26}H_{44}O_5$, monoacetate of $C_{26}H_{42}O_4$: C, 73.00; H, 9.64. Found: C, 72.69; H, 9.70.

Hydrolysis of a specimen of the acetate derivative of Compound C in 90% methanol containing 5% potassium hydroxide at room temperature for twenty-four hours gave a product which crystallized in long white needles from ethyl acetate; it melted at 260°.

Compound D.—Fraction Ib2a (1.99 g.) was dissolved in 50 cc. of dry ether and filtered through 12 g. of aluminum oxide; it was washed with 20 cc. of dry ether. The ether filtrate on evaporation gave 1.23 g. of a glass-like residue which was next dissolved in 35 cc. of an ether–petroleum ether (1:1) mixture and filtered through 12 g. of aluminum oxide; it was washed with 65 cc. of the solvent mixture. There was obtained on evaporation of the filtrate, 0.94 g. of residue which on treatment with ethyl acetate yielded 75.5 mg. of crystalline material, m. p. 198–217°.

Fraction Ib1a (2.7 g.) was dissolved in 40 cc. of carbon tetrachloride (containing 3% ethanol) and filtered through 12 g. of aluminum oxide; it was washed with 10 cc. of the solvent mixture. There was obtained on evaporation of the filtrate, 1.67 g. of residue which on treatment with ethyl acetate gave 155 mg. of crystalline material, m. p.

(17) Kindly furnished by Prof. O. Wintersteiner.

180–185°. It was recrystallized twice from ethyl acetate to give 70 mg., m. p. 190–195°; this product appeared to be a complex crystalline mixture. It was combined with the 75.5 mg. from Fraction Ib2a, which appeared to be of a similar nature and acetylated in the usual manner. There was obtained in all 166 mg. of acetylated material which was dissolved in 50 cc. of carbon tetrachloride and chromatographed over 12 g. of aluminum oxide.

TABLE IV

No. of fraction	Eluent	Vol., cc.	Eluate, mg.	Crystallized once from methanol, Mg.	M. p., °C.
1	CCl_4	60	7		
2	CCl_4	40	64	45	110–111
3	CCl_4	40	10	5	180–185
4	CCl_4	40	3		
5	Ethanol	40	80	38	150–160

Fraction 5 was a crystalline mixture which could not be resolved. Fraction 2, on repeated crystallization from methanol, yielded 28 mg. of needles, m. p. 111°; $[\alpha]^{18}D$ +72° (*c* 0.806 in absolute ethanol).

Anal. Calcd. for $C_{26}H_{42}O_4$: C, 74.61; H, 10.11; mol. wt., 419. Found: C, 74.36; H, 10.09; mol. wt., 408 (by titration of acetyl groups).

To 16.1 mg. (0.0385 ml.) of the acetate derivative, there was added 15 cc. of methanol and an excess of aqueous standard alkali. The solution was refluxed for four hours and then permitted to stand for forty-eight hours at room temperature. Back titration with standard acid solution indicated that 0.0394 milliequivalent of alkali had been neutralized, after deducting the value of the blank. Refluxing for an additional three hours did not cause any further neutralization of alkali. The hydrolyzate yielded a product which on crystallization from methanol gave 7 mg. of plates, m. p. 229–231°. Recrystallization from the same solvent gave 2 mg., melting sharply at 232–233° (Compound D). The empirical formula of Compound D is assumed to be $C_{24}H_{40}O_3$ since its acetate derivative appears to contain but one acetyl group.

TABLE V

No. of fraction	Eluent	Eluate, mg.	Hydrolyzate[18] mg.	Crystallized once from aqueous methanol, Mg.	M. p., °C.
1	Pentane:benzene (3:1) 105 cc.	293	263	6 (prisms)	202–207
2	Pentane:benzene (3:1) 25 cc.				
	Pentane:benzene (1:1) 90 cc.	79.7	70	29.5 (needles)	184–191
3	Benzene 50 cc.	79.6	68	24 (needles)	188–190

Compound E.—Fraction IIa (11.6 g.) was taken up in a little benzene and an excess of petroleum ether added. The precipitate (6.19 g.) was treated with Girard reagent T[19] to yield 3.58 g. of non-ketones. Treatment with ethyl acetate gave 448 mg. of crystals, m. p. 192–200°; these were difficultly soluble in ethyl acetate, acetone, benzene and chloroform but moderately soluble in ethanol. Recrystallization twice from ethanol gave 99 mg. of compact prisms, m. p. 204–206°; $[\alpha]^{26}D$ +37° (*c* 0.979 in ethanol). The material was recovered and crystallized repeatedly from alcohol but the melting point of each new crop rose until 11 mg. was obtained which melted at 216–218°. The original crystalline fraction was obviously a mixture. It was recovered (total, 433 mg.) and acetylated in the usual manner. The acetates (569 mg.) were dissolved in 40 cc. of a pentane: benzene (3:1) mixture and chromatographed over 10 g. of aluminum oxide (Table V).

The hydrolyzates of Fractions 2 and 3 were combined (total-138 mg.) and extracted with small portions of hot benzene, using in all 35 cc. of benzene. A residue of 51.5 mg. remained. The benzene extract was chromatographed over 7.5 g. of aluminum oxide.

TABLE VI

No. of fraction	Eluent	Vol., cc.	Eluate, mg.	Crystallized once from aqueous methanol, Mg.	M. p., °C.
1	Benzene	175	trace		
2	Benzene (4% ethanol)	25	34.4 (semicryst.)	14.0	195–196
3	Benzene (4% ethanol)	25	23.1 (cryst.)	14.9	198–199
4	Benzene (4% ethanol)	25	4.4 (oil)		
5	Benzene (4% ethanol)	25	0.8		
6	Benzene (8% ethanol)	50	21	10.7	210–215

Fractions 2 and 3 were crystallized repeatedly from aqueous methanol to give 8.5 mg. of needles, m. p. 202° (Compound E).

Anal. Calcd. for $C_{24}H_{42}O_4$: C, 73.04; H, 10.72; mol. wt., 395. Found: C, 72.66; H, 10.51; mol. wt. (Rast), 380, 395.

The mother liquors of Compound E were worked up to give an additional 11.6 mg., m. p. 199–200°. It was acetylated in the usual manner to give on repeated crystallization from aqueous methanol, 4.7 mg. of needles, m. p. 142.5°.

Anal. Calcd. for $C_{28}H_{46}O_5$, diacetate of Compound E: C, 70.25; H, 9.69. Found: C, 70.36; H, 9.56.

The carbon and hydrogen analyses and the molecular weight (Rast) determinations were performed by Mr. Wm. Saschek at Columbia University.

Summary

An exploratory examination of the neutral, non-saponifiable fraction of ox bile yielded five compounds (other than cholesterol) in small amount. Tentative empirical formulas were established for these, pointing to a steroid constitution. Structural elucidation must, however, wait until more material is available.

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(18) Hydrolyzed in 90% methanol containing 5% potassium hydroxide at room temperature for twenty-four hours.

(19) A. Girard and G. Sandulesco, *Helv. Chim. Acta*, **19**, 1095 (1936).