hydrolysis, presumably as a result of the presence of the double lactol ring system.[24] The structural similarity of compound II with sedoheptulosan is further reflected in the similar R_f values obtained with several different solvent systems.

It has been demonstrated that the ketohexose, fructose, under the influence of HCl, is partially converted to two di-D-fructose dianhydrides.[25] The possibility of compound II being, in a similar manner, di-D-*altro*-heptulose dianhydride is excluded since several moles of periodate should be readily consumed. Conclusive proof of the structure of compound II must await the production of sufficient material for degradative studies, and the characterization of derivatives.

The formation of compound VI is amenable to explanation since the production of analogous compounds by the acid treatment of hexoses and pentoses is well known.[26] The absorption spectrum of compound VI is almost identical with that of 5-(hydroxymethyl)-2-furfuraldehyde. This spectrum is at variance with the absorption spectrum of furfural owing to substitution in the 5-position. Although the absorption spectrum indicates that the furan ring is still intact in compounds VI, the non-formation of formaldehyde on treatment with strong acid indicates that the absorption is not caused by 5-(hydroxymethyl)-2-furfuraldehyde. This is also shown by the comparative absorption spectra of glucose, ribose, sedoheptulosan and compound VI when reacting with orcinol. On the basis of the analogous reaction of acid on fructose[27] this compound would then be 5-(1,2-dihydroxyethyl)-2-furfuraldehyde. This compound contains a pair of *cis*-hydroxyl groups (or at least potentially of *cis* configuration) thereby explaining the affinity of the borate complex for the anion-exchange resin. The presence of the furan ring is shown by the obtained absorption spectrum.

The absorption spectrum of compound VI with orcinol exhibits the same maximum as does sedoheptulosan. It thus appears that the formation of compound VI is a requisite for the orcinol reaction of D-*altro*-heptulose and sedoheptulosan since compound VI is formed irreversibly from sedoheptulosan. The non-linearity of the orcinol reaction with larger amounts of sedoheptulosan is probably attributable to the cleavage of furan compounds in acid media. The formation of furfural from 5-(1,2-dihydroxyethyl)-2-furfuraldehyde is indicated by the increased absorption at 670 mμ with longer times of heating for the orcinol reaction. Pretreatment of sedoheptulosan with HCl before carrying out the orcinol reaction has been reported by Dische,[28] who has also applied the cysteine and diphenylamine reactions to the quantitative determination of sedoheptulosan. These latter reactions appear to be more applicable to the determination of larger amounts of sedoheptulosan. The non-linearity of the orcinol reaction with sedoheptulose (above *ca.* 12 μg./ml.) has been reported.[29]

Acknowledgment.—The authors wish to thank Dr. Nelson K. Richtmyer and Dr. James W. Pratt of the National Institutes of Health for suggesting the structure of compound II.

(24) R. J. Dimler, "Advances in Carbohydrate Chemistry," Academic Press, Inc., New York, N. Y., Vol. 7, 1952, p. 37.

(25) M. L. Wolfrom and M. G. Blair, THIS JOURNAL, **70**, 2406 (1948).

(26) A. P. Dunlop and F. N. Peters, "The Furans," Reinhold Publ. Corp., New York, N. Y., 1953.

(27) W. N. Haworth and W. G. M. Jones, *J. Chem. Soc.*, 65 (1944).

(28) Z. Dische, *J. Biol. Chem.*, **204**, 983 (1953).

(29) A. Nordal and R. Klevstrand, *Anal. Chim. Acta*, **4**, 411 (1950).

OAK RIDGE, TENNESSEE

[CONTRIBUTION FROM THE PHARMACEUTICAL INSTITUTE, MEDICAL FACULTY, UNIVERSITY OF KYUSHU]

Cholesterol and Related Compounds. I. Structure of a New Non-conjugated Cholestadienol from 7-Bromocholesterol

BY KYOSUKE TSUDA, KO ARIMA[1] AND RYOICHI HAYATSU[1]

RECEIVED DECEMBER 28, 1953

Treatment of 7α- and 7β-bromocholesteryl acetate (Ia) (benzoate, Ib) with pyridine or other bases gives, as the chief reaction product, $\Delta^{5,8(9)}$-cholestadienyl acetate (IIa) (benzoate IIb). Nitric acid oxidation of IIa gives methylpyromellitic acid (VI) while hydrogenation of IIa in ethyl acetate in the presence of platinum oxide gives $\Delta^{8(9)}$-cholestenyl acetate (III). Reduction of III with platinum oxide in acetic acid yields cholestanyl acetate (V). Chromic acid oxidation of IIa gives 7-keto-$\Delta^{5,8(9)}$-cholestadien-3β-ol acetate (VIII) which on catalytic hydrogenation with palladium is converted into 7-keto-cholestan-3β-ol acetate (IX). Dienone–phenol rearrangement of VIII with acetic anhydride and sulfuric acid gives a steroidal phenol (Xb) whose methyl ether (Xc) forms methylnitrobenzenetetracarboxylic acid (XI) by oxidation with nitric acid and an anthracene series hydrocarbon, $C_{19}H_{18}$ (XIIa and b), by dehydration with selenium.

A. E. Bide, *et al.*,[2] obtained $\Delta^{4,6}$-cholestadienyl acetate by treating 7α-bromocholesteryl acetate[3] (Ia) with 2,6-lutidine, and $\Delta^{4,6}$-cholestadienyl acetate and 7-dehydrocholesteryl acetate by the treatment of Ia with diethylaniline. H. Schalteggar, *et al.*,[4] obtained 7-dehydrocholesteryl benzoate by the treatment of 7α-bromocholesteryl benzoate (Ib) with dimethylaniline. One of the authors, Arima,[5] treated Ia with ammonium thiocyanate and obtained a new, non-conjugated dienol of m.p. 147–148°. The present paper describes the detailed examinations of conditions for formation of

(1) Takamine Research Laboratory, Sankyo Co., Ltd., Tokyo, Japan.

(2) A. E. Bide, H. B. Henbest, E. R. H. Jones, R. W. Peevers and P. A. Wilkinson, *J. Chem. Soc.*, 1783 (1948).

(3) In the present paper the more dextrorotatory isomer is named "7β."

(4) H. Schalteggar and F. X. Mullner, *Helv. Chim. Acta*, **34**, 1096 (1951).

(5) K. Arima, *Pharm. Bull. (Japan)*, **1**, 224 (1953), *et seq.*

TABLE I

PRODUCTS OF THE REACTION OF 7α- OR 7β-BROMOCHOLESTERYL BENZOATE WITH BASE

7-Br compd.	Base	React. condn.	Product, % Non-conjugated dienol benzoate (II)	7-Dehydrocholesteryl benzoate (VII)	$\Delta^{4,6}$-Cholestadienol benzoate	Cholesteryl benzoate[a]	7-Hydroxycholesteryl benzoate
α, β	Pyridine	100°, 5 hr.	80[b]	4[c]	6[c]		
α	*sym*-Collidine	Room temp. 10 hr.	35	4–5[c]	6–8[c]	5[d]	
	γ-Picoline, α-picoline		40[d]				
α	NH_4SCN,[e] acetone	Room temp.	80[b]				
α	Pyridine, *p*-toluenesulfonic acid Ag salt	Room temp., 24 hr.	70[b]				
α	AgOH[f]	Room temp., 12 hr.	40[d]				30 (7β)[d] 3 (7α)[d]

[a] Formed on reaction with *sym*-collidine. This reaction mechanism is assumed to be analogous to the formation of cholestanone from 2-iodocholestanone (*cf.* G. Rosenkranz, *et al.*, THIS JOURNAL, **72**, 4078 (1950)). [b] Recrystallized. [c] Ultraviolet spectral analysis of the crude product: calcd. from the extinction of the bands 239 mμ ($\Delta^{4,6}$-dienol), 272 and 282 mμ (7-dehydro). [d] Chromatographic analysis. [e] *Cf.* K. Arima, *Pharm. Bull.* (*Japan*), 1, 224 (1953). [f] H. Schaltegger (*Helv. Chim. Acta*, **34**, 1096 (1951)) carried out this reaction and chromatographic analysis on the product. The substance described as structurally unknown compound X in this report seems to be identical with the non-conjugated dienol obtained in the present experiments, as evidenced by the chromatographic adsorbability.

TABLE II

COMPARISON OF NON-CONJUGATED DIENOL (II), 7-DEHYDROCHOLESTEROL (VII) AND ISODEHYDROCHOLESTEROL (IV)

	M.p., °C.	α_D	Max., mμ	Salkowsky color test	Acetate, m.p., °C.	α_D	Benzoate, m.p., °C.
II	147–148	−54.7°	Nil	Indigo blue	121–122	−48.3°	149–150
VII[a]	146–148	−114	282	Bluish violet	129–130	−77.7	140–141
IV[b]	120–122	−17.9	275	.....	111–112	−10.7	146

[a] A. Windaus, *et al.*, *Ann.*, **534**, 122 (1938). [b] S. Bernstein, *et al.*, *J. Org. Chem.*, 14, 433 (1949).

this non-conjugated dienol and studies on its chemical structure.

Both the stable 7α-type and the labile 7β-type[3] of 7-halocholesteryl benzoate react in a similar manner with primary and secondary amines. 7α-Bromocholesteryl benzoate gave 7β-anilino derivative,[6] m.p. 188–189°, α_D + 134.5°, and 7β-piperidino derivative,[6] m.p. 166–168°, α_D +92.8°. 7β-Bromocholesteryl benzoate gave the epimeric 7α-anilino derivative, m.p. 174–175°, α_D +120° and the 7α-piperidino derivative, m.p. 195–197°, α_D +52°. However, the reaction of the tertiary amine, pyridine, differs with 7α- and 7β-compounds. When the 7α-bromo compound is allowed to stand with cold pyridine it is converted to a pyridinium salt, m.p. 189–190°, while the same treatment of the 7β-bromo compound gives the non-conjugated dienol. Further heating of the pyridinium salt of 7α-bromocholesteryl benzoate with pyridine finally affords the non-conjugated dienol. The yield of the non-conjugated dienol is 80% when 7α- or 7β-bromocholesteryl benzoate is heated with pyridine, and a minute amount of 7-dehydrocholesteryl benzoate and $\Delta^{4,6}$-cholestadienyl benzoate are obtained as by-products. When 7-bromocholesteryl benzoate is treated with 2,4,6-collidine, α-picoline, γ-picoline, silver hydroxide or an aqueous solution of *p*-toluenesulfonic acid, the chief reaction product is the non-conjugated dienol. The yield and by-products obtained are given in Table I.

This non-conjugated dienol is an isomer of 7-dehydrocholesterol and isodehydrocholesterol and differs from these substances in that it does not show any absorption band in the region of 230–360 mμ in the ultraviolet spectrum. Comparison of these isomers is shown in Table II.

(6) K. J. Knox and S. Bernstein, *J. Org. Chem.*, **16**, 1069 (1951).

Catalytic reduction of the non-conjugated dienol acetate (IIa) in ethyl acetate with platinum oxide results in the absorption of one mole of hydrogen to give a substance melting at 124–126°, α_D +33.5°, which was found to be identical with $\Delta^{8(9)}$-cholesten-3β-ol acetate (III), prepared from isodecholesterol (IV) according to the method of H. Wieland, *et al.*,[7] and D. H. R. Barton, *et al.*[8] Catalytic reduction of III in glacial acetic acid with platinum oxide, at 80°, results in absorption of one mole of hydrogen to give cholestanyl acetate (V). IIa gives methylbenzenetetracarboxylic acid (tetramethyl ester, m.p. 122–123°) by oxidation with nitric acid. This tetramethyl ester failed to show any depression of the melting point on admixture with the methyl ester, m.p. 122–123°, of a carboxylic acid obtained by the nitric acid oxidation of 7-dehydrocholesterol.[9] It is known that this carboxylic acid is methylpyromellitic acid (VI)[10] and that it is formed by nitric acid oxidation only when two double bonds are present in the B ring, as in 7-dehydrocholesterol, isodehydrocholesterol and ergosterol.[10] Considering the results of hydrogenation, it may be assumed that the two non-conjugated double bonds in II are at $\Delta^{5,8(9)}$.

Chromic acid oxidation of IIa gives an α,β-unsaturated ketone VIII, m.p. 151–152°, λ_{max}^{EtOH} 238 mμ, α_D −67.3°, which forms a semicarbazone of m.p. 210–212° dec., $\lambda_{max}^{Et_2O}$ 281 mμ. Catalytic reduction of VIII in acetic acid with palladium easily gives

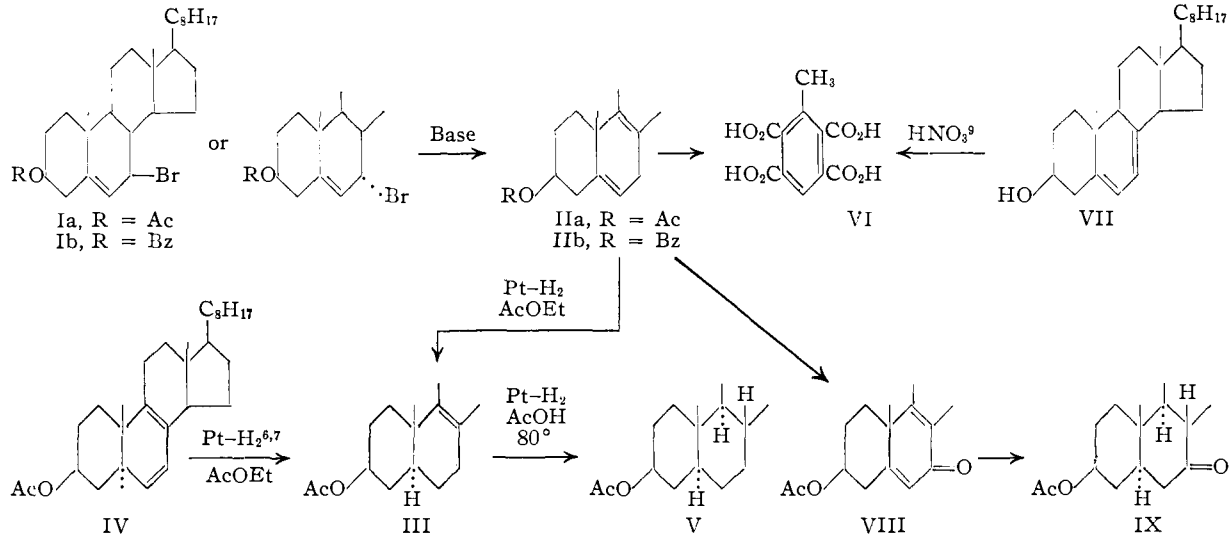
(7) H. Wieland and W. Benend, *Ann.*, **554**, 1 (1943).

(8) D. H. R. Barton and J. D. Cox, *J. Chem. Soc.*, 214 (1949).

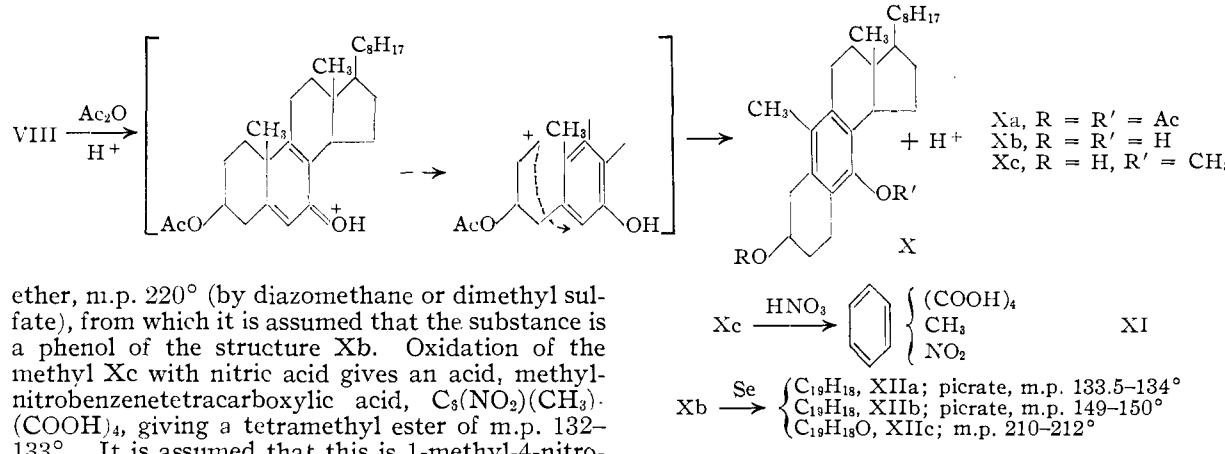
(9) L. F. Fieser and Mary Fieser, "Natural Products Related to Phenanthrene," Reinhold Publ. Corp., New York, N. Y., 1949, pp. 160 and 169. No evidence is available concerning the mechanism of formation of methylpyromellitic acid.

(10) K. Alder and B. Kruger, *Ber.*, **86**, 985 (1953).

7-ketocholestanyl acetate (IX), from which it can be seen that the double bond at $\Delta^{8(9)}$ has become easily reducible by the influence of the carbonyl group.[11] The transformation establishes the position of the carbonyl group in VIII and confirms the structure II. The structure VIII is further supported by the fact the substance easily undergoes the dienone–phenol rearrangement.[12]



In this reaction, VIII, is dissolved in a mixture of chloroform and acetic anhydride and a mixture of sulfuric acid and acetic anhydride is added; the color of the solution changes from yellow, through pink and green, to dark green, and a diacetyl compound Xa, m.p. 164°, is obtained as the reaction product. Saponification of this compound gives a substance of m.p. 263–265°, corresponding to $C_{27}H_{42}O_2$, showing $\lambda_{max}^{Et_2O}$ 275 mμ. The substance dissolves in hot caustic alkali and gives a methyl ether, m.p. 220° (by diazomethane or dimethyl sulfate), from which it is assumed that the substance is a phenol of the structure Xb. Oxidation of the methyl Xc with nitric acid gives an acid, methylnitrobenzenetetracarboxylic acid, $C_6(NO_2)(CH_3)\cdot(COOH)_4$, giving a tetramethyl ester of m.p. 132–133°. It is assumed that this is 1-methyl-4-nitrobenzene-2,3,5,6-tetracarboxylic acid formed by the nucleophilic substitution of the methoxyl by a nitro group.



Selenium dehydrogenation of Xb and chromatographic purification of the product through an alumina column gives an easily crystallizable substance (XIIc), m.p. 210–212°, $C_{19}H_{18}O$, and an oily hydrocarbon, which can be converted to a crystalline picrate but does not easily crystallize on regeneration. Both hydrocarbons (XIIa and XIIIb) correspond to the formula $C_{19}H_{18}$, and give picrates of m.p. 133.5–134° and 149–150°. This composition suggests an aromatic hydrocarbon. The ultraviolet absorption curves of these hydrocarbons were compared with those of hydrocarbons of the anthracene and phenanthrene series (Fig. 1). Phenanthrenic hydrocarbons show the first peak at 250–260 mμ and the second peak at 270–280 mμ, while anthracenic hydrocarbons show the first peak at 250–260 mμ and the second peak at 330–350 mμ.[13] Both XIIa and XIIb clearly show absorption curves of the anthracenic type and, therefore, the cyclopentanoanthracene nucleus (X) can be assumed for the phenol. D. H. Barton, *et al.*,[14] have carried out the dienone–phenol rearrangement of 7-keto-$\Delta^{5,8(9)}$-lanostadienyl acetate but made no experiments to confirm the structure of the phenol formed.

(11) L. F. Fieser and J. E. Herz, THIS JOURNAL, **75**, 121 (1953).

(12) A. L. Wilds and C. Djerassi, *ibid.*, **68**, 1712 (1946); R. B. Woodward and J. Singh, *ibid.*, **72**, 494 (1950); C. Djerassi and G. Rosenkranz, *ibid.*, **72**, 4542 (1950); A. Dreiding, W. J. Pummer and A. T. Tomasewski, *ibid.*, **75**, 3159 (1953); H. H. Inhoffen, G. Kolling and P. Nehring, *Ber.*, **85**, 89 (1952); R. B. Woodward, H. H. Inhoffen, H. O. Larson and K. H. Menzel, *ibid.*, **86**, 594 (1953).

(13) R. A. Friede and M. Orchin, "Ultraviolet Spectra of Aromatic Compounds," John Wiley and Sons, Inc., New York, N. Y., 1951.

(14) D. H. Barton and B. R. Thomas, *J. Chem. Soc.*, 1842 (1953).

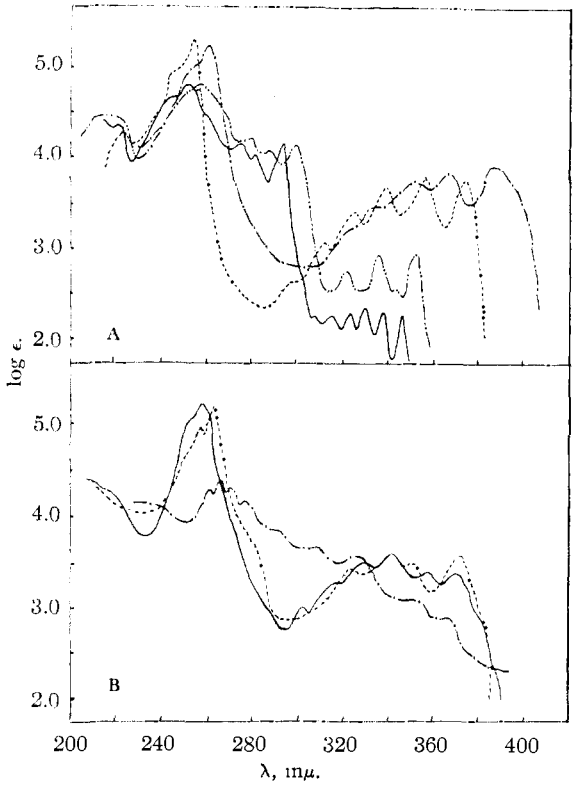


Fig. 1.—Ultraviolet absorption spectra, A: ..., anthracene; -·-·-, 4H-methylbenzanthracene; ————, phenanthracene; —·—, cyclopentenophenanthrene. B: ————, XIIa; ..., XIIb; —·—·—, XIIc.

Experimental

$\Delta^{5,8(9)}$-Cholestadienyl Benzoate (IIb).—(1) A solution of 55 g. of 7α-bromocholesteryl benzoate,[15] m.p. 143–144°, $\alpha^{22}D$ −179.5° (*c* 1.47, $CHCl_3$), dissolved in 150 cc. of pyridine was heated at 100° for 5 hours, and the reaction mixture was poured slowly into a large amount of water acidified with diluted hydrochloric acid, under ice-chilling. The reddish-brown oil that separated solidified on standing, and the product was collected, washed with 10% hydrochloric acid and a small amount of methanol and acetone, dried, and recrystallized from a mixture of chloroform and acetone to colorless prisms, m.p. 149–150°; yield, 36.5 g.; soluble in chloroform, benzene and ether, sparingly soluble in acetone and alcohol.

Anal. Calcd. for $C_{34}H_{48}O_2$: C, 83.54; H, 9.81. Found: C, 83.47; H, 10.20.

The same result was obtained when 7β-bromocholesteryl benzoate[15] m.p. 115° dec., $\alpha^{22}D$ +16° (*c* 1.2, $CHCl_3$), was treated in the same manner.

(2) A brei-like mixture of 10 g. of 7α-bromocholesteryl benzoate and 20 cc. of α- or γ-picoline was allowed to stand overnight and treated exactly in the same manner as in the foregoing (1) when IIb was obtained in 35–45% yield. When *sym*-collidine was used, the crude IIb obtained was saponified and then acetylated, and the product was chromatographically purified, through alumina, as described later. The initial fraction furnished $\Delta^{5,8(9)}$-cholestadienyl acetate (IIa) in 35% yield and later fractions gave crystals of m.p. 113–114°. When these crystals were refluxed with 5% methanolic potassium hydroxide, cholesterol, m.p. 146–148°, $\alpha^{23}D$ −38.5°, was obtained indicating the crystals to be those of cholesteryl acetate; yield approximately 5%.

(3) A mixture of 15 g. of 7α-bromocholesteryl benzoate and silver hydroxide freshly prepared from 9 g. of silver nitrate, in 50 cc. of ether, was shaken for 16 hours. After filtration, ether was removed by distillation and the oily residue was dissolved in 500 cc. of a 1:1 mixture of petroleum ether and benzene and passed through a column containing 360 g. of alumina. The column was eluted with 8 liters of the same solvent and the effluent was fractionated into 500-cc. portions. Fractions 1–3 gave crystals of m.p. 144–146° which recrystallized from a chloroform–acetone mixture to IIb, m.p. 149–150°, yield 4.32 g. Fraction 4 gave crystals of m.p. 139–141°, and fractions 5–9, crystals of m.p. 182–185°, which recrystallized from acetone to 7β-hydroxycholesteryl benzoate, m.p. 189–190°,[4] yield 3.8 g. Fractions 12–16 furnished crystals of m.p. 162–165° which recrystallized from acetone to 7α-hydroxycholesteryl benzoate, m.p. 165–167°,[4] yield 357 mg.

(4) Five grams of 7α-bromocholesteryl benzoate was dissolved in 50 cc. of pyridine, 7 g. of silver *p*-toluenesulfonate was added, and the mixture was allowed to stand overnight. After filtration, the filtrate was poured into dilute hydrochloric acid solution, and the precipitate was collected by filtration. The precipitate was washed with 10% hydrochloric acid and water, extracted with ether, and the ethereal residue was recrystallized from a chloroform–acetone mixture to IIb, m.p. 149–150°, yield 2.6 g.

In exactly the same manner, 5 g. of 7β-bromocholesteryl benzoate furnished 2.5 g. of IIb.

$\Delta^{5,8(9)}$-Cholestadien-3β-ol.—Thirty grams of IIb was dissolved in the smallest possible amount of benzene, methanolic potassium hydroxide (400 cc. of methanol + 5 g. of potassium hydroxide) added, and the mixture was refluxed for 3 hours. A small amount of water was added, and the mixture allowed to stand at 0° when crystals separated. The crystals were collected by filtration and recrystallized from a chloroform–acetone mixture to colorless needles, m.p. 147–148°, $\alpha^{25}D$ −54.7° (*c* 1.17, $CHCl_3$); soluble in chloroform and benzene, easily soluble in hot acetone but sparingly so in cold, sparingly soluble in alcohol; yield 18.4 g.

Anal. Calcd. for $C_{27}H_{44}O$: C, 83.85; H, 11.45. Found: C, 83.70; H, 11.77.

$\Delta^{5,8(9)}$-Cholestadienyl acetate (IIa), obtained by allowing a mixture of the dienol, pyridine and acetic anhydride to stand overnight, or by treatment of 7α-bromocholesteryl acetate as in the case of benzoate, recrystallized from acetone as colorless needles, m.p. 121–122°, $\alpha^{25}D$ −48.3° (*c* 3.31, $CHCl_3$).

Formation of a Salt from 7α-Bromocholesteryl Benzoate and Pyridine.—A mixture of 10 g. of 7α-bromocholesteryl benzoate and 40 cc. of pyridine was allowed to stand for 7 hours at room temperature. This was poured into dilute hydrochloric acid and the crystals that precipitated were collected by filtration. After drying, they were recrystallized from a chloroform–ether mixture to colorless needles, m.p. 189–190°, $\alpha^{20}D$ +71° (*c* 1.02, $CHCl_3$), yield 3.2 g.

Anal. Calcd. for $C_{39}H_{54}O_2BrN$: C, 72.22; H, 8.33; N, 2.16. Found: C, 72.47; H, 8.51; N, 2.07.

7α-Anilinocholesteryl Benzoate.—A brei-like mixture of 3 g. of 7β-bromocholesteryl benzoate and 6 cc. of aniline was allowed to stand overnight, poured into dilute hydrochloric acid, and the precipitated crystals were collected by filtration. The crystals were washed consecutively with water, dilute sodium hydroxide, water, and methanol and recrystallized from chloroform to colorless prisms, m.p. 174–175°, $\alpha^{25}D$ +120° (*c* 1.07, $CHCl_3$), yield 1.7 g.

Anal. Calcd. for $C_{40}H_{55}O_2N$: C, 82.61; H, 9.46; N, 2.41. Found: C, 82.79; H, 9.88; N, 2.50.

7β-Anilinocholesteryl Benzoate.—Similar treatment of a mixture of 3 g. of 7α-bromocholesteryl benzoate and 6 cc. of aniline yielded 2.2 g. of colorless prisms, m.p. 188–189°, $\alpha^{25}D$ +134.5° (*c* 1.22, $CHCl_3$), the data agree well with the results obtained by Knox and Bernstein.[6]

Anal. Calcd. for $C_{40}H_{55}O_2N$: C, 82.61; H, 9.41; N, 2.41. Found: C, 82.40; H, 9.72; N, 2.38.

7α-Piperidinocholesteryl Benzoate.—A mixture of 2 g. of 7β-cholesteryl benzoate, 2 cc. of piperidine and 20 cc. of toluene was heated for 10 minutes at 90–95°, the mixture was poured into a dilute hydrochloric acid, and the colorless crystals of the hydrochloride that precipitated were collected by filtration. After washing with water, the crystals were suspended in ether, shaken with 10% sodium hydroxide and the ether layer was separated. After washing with water and drying, the ether was removed by filtration and the residue was recrystallized from a mixture of benzene and acetone to colorless needles, m.p. 195–197°, $\alpha^{25}D$ +52° (*c* 1.87, $CHCl_3$), yield 370 mg.

(15) H. Schaltegger, *Helv. Chim. Acta*, **33**, 2101 (1950).

Anal. Calcd. for $C_{39}H_{59}O_2N$: C, 83.42; H, 10.48; N, 2.44. Found: C, 83.70; H, 10.29; N, 2.42.

7β-Piperidinocholesteryl Benzoate.—A mixture of 2 g. of 7α-cholesteryl benzoate, 2 cc. of piperidine and 20 cc. of toluene was treated as in the foregoing and yielded 410 mg. of colorless needles, m.p. 166–168°, $\alpha^{25}D$ +92.8° (*c* 2.02, $CHCl_3$).

Anal. Calcd. for $C_{39}H_{59}O_2N$: C, 83.42; H, 10.48; N, 2.44. Found: C, 83.67; H, 10.32; N, 2.44.

These results agree well with those reported.[6]

Hydrogenation of $\Delta^{5,8(9)}$-Cholestadienyl Acetate (IIa).—A solution of 5 g. of IIa in 40 cc. of ethyl acetate, was shaken with 300 mg. of platinum oxide and hydrogen; one mole of hydrogen was absorbed in 1.5 hours. After filtration, the filtrate was distilled under diminished pressure and the solid residue was recrystallized from acetone to crystals melting at 108–112°. A solution of this substance in 100 cc. of petroleum ether was passed through a column of 80 g. of alumina, and the column was developed with 500 cc. of petroleum ether. The effluent was divided into fractions of 60 cc. each. Fractions 3–4 yielded crystals of m.p. 117–118° which recrystallized from ethyl acetate–methanol to colorless needles, m.p. 124–126°, $\alpha^{23}D$ +33.5° (*c* 1.08, $CHCl_3$). No depression of the melting point occurred on admixture with an authentic sample of $\Delta^{8(9)}$-cholestenyl acetate[7,8] (III) prepared by the hydrogenation of isodehydrocholesterol (IV). Fractions 7–9 yielded crystals of m.p. 107–109°, $\alpha^{23}D$ +14.6° (*c* 0.978, $CHCl_3$) which, when refluxed with 5% methanolic potassium hydroxide for 2 hours gave crystals of m.p. 128–129°, $\alpha^{23}D$ +51.6° (*c* 1.26, $CHCl_3$); the constants agree with those of $\Delta^{8(9)}$-cholestenol, hence the crystals from fractions 7–9 were crude III.

Anal. Calcd. for $C_{29}H_{48}O_2$: C, 81.30; H, 11.21. Found: C, 81.52; H, 11.37.

Three grams of III, dissolved in 40 cc. of acetic acid, was hydrogenated at 80° with 0.3 g. of platinum oxide. Two-thirds of a mole of hydrogen was absorbed in 3 hours after which 100 mg. of platinum oxide was added, hydrogenation continued, and one mole of hydrogen was absorbed during 12 hours. After filtration, the filtrate was evaporated under diminished pressure, and the residue was recrystallized three times from acetone to crystals of m.p. 113–114°, yield 1.2 g. No depression of the melting point occurred on admixture of this substance with an authentic sample of cholestanyl acetate (V).

Nitric Acid Oxidation of $\Delta^{5,8(9)}$-Cholestadienyl Acetate (IIa).—A mixture of 3 g. of IIa and 30 cc. of nitric acid (d. 1.38) was heated at 100° for 30 hours with shaking. After completion of the reaction, nitric acid was distilled off under diminished pressure and the residue was dried in a desiccator. To this was added 10 times the calculated amount of diazomethane in ether and the mixture was allowed to stand overnight. Ether was then evaporated under diminished pressure and the residue was recrystallized from alcohol to colorless needles, m.p. 122–123°, yield 72 mg. Admixture with methyl methylbenzenetetracarboxylate (VI), m.p. 122–123°, obtained by the same treatment of 7-dehydrocholesterol, showed no depression of the melting point.

Anal. Calcd. for $C_{15}H_{16}O_8$: C, 55.5; H, 4.9. Found: C, 55.30; H, 4.82.

7-Keto-$\Delta^{5,8(9)}$-cholestadien-3β-ol Acetate (VIII).—To a solution of 52 g. of IIa dissolved in 600 cc. of glacial acetic acid, being stirred at 45–50°, 37 g. of chromic acid was added and stirring was continued for further 2 hours, and then 15 cc. of ethanol was added. The acetic acid solution was concentrated under diminished pressure to about 20 cc., 20 cc. of water was added, and the solution was allowed to stand at 0° overnight. The crystals that precipitated were collected by filtration and recrystallized from alcohol to colorless prisms, m.p. 151–152°, $\alpha^{22}D$ −67.3° (*c* 0.951, $CHCl_3$), λ_{max}^{EtOH} 238 mμ (log ε 4.475), yield 14.2 g.

Anal. Calcd. for $C_{29}H_{44}O_3$: C, 79.1; H, 10.0. Found: C, 79.42; H, 10.33.

Semicarbazone: m.p. 210–212° dec., $\lambda_{max}^{Et_2O}$ 281 mμ (log ε 4.012).

Anal. Calcd. for $C_{30}H_{47}O_3N_3$: N, 8.4. Found: N, 8.42.

Hydrogenation of 7-Keto-$\Delta^{5,8(9)}$-cholestadien-3β-ol Acetate (VIII).—A solution of 0.3 g. of VIII in 30 cc. of glacial acetic acid, with 200 mg. of 10% palladium–carbon, was shaken in a hydrogen stream. After absorption of about two moles of hydrogen, the mixture was filtered, water was added to the filtrate, and the solid that separated was collected by filtration. The solid was recrystallized from methanol to colorless scaly crystals, m.p. 146–148°, which showed no depression on admixture with an authentic sample of 7-ketocholestanyl acetate (IX).

Dienone–Phenol Rearrangement of 7-Keto-$\Delta^{5,8(9)}$-cholestadien-3β-ol Acetate (VIII).—To a solution of 8 g. of VIII in a mixture of 10 cc. of chloroform and 180 cc. of acetic anhydride, a mixture of 4 cc. of concentrated sulfuric acid and 40 cc. of acetic anhydride was added, when the color of the solution changed from yellow, through pink and green, to dark green. After maintaining the mixture at 20° for 6 hours, ice-water was added to the solution under stirring, and the acid was neutralized by the dropwise addition of sodium carbonate solution under chilling. After allowing the mixture to stand at 0° overnight, the supernatant clear solution was decanted, and the oily precipitate was taken up in ether. The ether layer was washed with water, dried, and the ether evaporated under reduced pressure. When the residual oil was allowed to stand at −20° for 10 hours, it solidified but this acetate was difficult to purify further. This acetate (Xa) was refluxed for 2 hours with 5% alcoholic potassium hydroxide, acidified with diluted hydrochloric acid, and extracted with ether. After washing with water and drying, the ether was distilled under a low pressure, and the pale yellow powder thereby obtained was recrystallized from alcohol to colorless needles, m.p. 261–265°, $\lambda_{max}^{Et_2O}$ 275 mμ (log ε 3.789), yield 3.2 g., insoluble in cold potassium hydroxide but soluble in hot alkali.

Anal. Calcd. for $C_{27}H_{42}O_2$: C, 81.40; H, 10.55. Found: C, 81.47; H, 10.38.

Two grams of this steroidal phenol (Xb) was dissolved in 50 cc. of alcohol, 7.2 cc. of a solution of 6 g. of sodium hydroxide in 10 cc. of water was added, and, while stirring this solution, 10 cc. of dimethyl sulfate added. This procedure was repeated four times, the mixture was warmed at 60° for 30 minutes, and ice-water was added to the mixture. The powder that separated was collected by filtration and repeatedly recrystallized from alcohol to colorless prisms (Xc), m.p. 218–220°, yield 1.1 g. Xb does give Xc by treatment with diazomethane but the yield is poor.

Anal. Calcd. for $C_{29}H_{44}O_2$: C, 82.07; H, 10.37. Found: C, 82.40; H, 10.73.

Nitric Acid Oxidation of Methyl Ether (Xc) of Steroidal Phenol.—A mixture of 2 g. of Xc and 15 cc. of nitric acid (d. 1.38) was heated for 30 hours at 100°. After cooling, this was evaporated to dryness under a diminished pressure and dried in a desiccator. The dried residue was dissolved in a small amount of alcohol, allowed to stand at 0° overnight, and pale yellow microcrystals (XI), m.p. 220–226° dec., were obtained; yield 130 mg.

Anal. Calcd. for $C_{11}H_7O_{10}N$: N, 4.47. Found: N, 4.93.

This substance was added to an ether solution of diazomethane and the product was recrystallized from alcohol to colorless needle crystals, m.p. 132–133°.

Anal. Calcd. for $C_{15}H_{15}O_{10}N$: C, 49.4; H, 4.01; N, 3.7. Found: C, 49.22; H, 4.43; N, 3.72.

Selenium Dehydrogenation of the Steroidal Phenol (Xb).—A mixture of 6 g. of Xb and 9 g. of selenium was heated for 2 hours at 290–300°, and for 32 hours at 330–340°. After cooling, the reaction mixture was extracted with benzene and the filtered solution distilled under reduced pressure to remove benzene. The residue was distilled at 0.002 mm. and 1.4 g. of a distillate boiling out at 170–250° was obtained.

The distillate was dissolved in 100 cc. of 1:1 of petroleum ether–benzene and passed through a column containing 60 g. of aluminum oxide. The column was developed with 400 cc. of the same solvent and the effluent was fractionated into 40-cc. portions: fractions 1–3 yielded an oily substance (crude XIIa), fractions 4–5, an oily substance (crude XIIb), and fractions 10–12 also oily substance (crude XIIc).

The crude, oily XIIa was dissolved in 20 cc. of petroleum ether, passed through 30 g. of alumina, and the column was eluted with 120 cc. of petroleum ether. The effluent was divided into fractions of 20 cc. each, and 400 mg. of the oily substance obtained from fractions 1–3 was dissolved in a small amount of benzene. To this was added a benzene solution of 500 mg. of picric acid and the mixture was

allowed to stand at 0° for 24 hours. The orange needles, m.p. 126–130°, that separated were again chromatographed through an alumina layer in benzene and a free hydrocarbon was obtained from the effluent. This hydrocarbon failed to crystallize but gave a picrate of orange needles, m.p. 133.5–134°, from alcohol (picrate of XIIa).

Anal. Calcd. for $C_{19}H_{18}$·$C_6H_3O_7N_3$: C, 63.15; H, 4.4; N, 8.8. Found: C, 63.52; H, 4.21; N, 8.66.

The oily hydrocarbon (XIIa), obtained by passing the benzene solution of the picrate of m.p. 133.5–134° through an alumina layer, was dried at a reduced pressure; λ_{max}^{EtOH} 260 mμ (Fig. 1).

Anal. Calcd. for $C_{19}H_{18}$: C, 92.65; H, 7.35. Found: C, 92.42; H, 7.62.

The crude XIIb was dissolved in 20 cc. of petroleum ether, passed through a column containing 30 g. of alumina, and the column was eluted with 120 cc. of petroleum ether. The effluent was fractionated into 20-cc. portions, and the oily substance obtained from fractions 2–4 was converted into a picrate. This picrate was also chromatographed through alumina as a benzene solution, as in the foregoing, and the free hydrocarbon obtained from the effluent was again converted to the picrate which recrystallized from alcohol to yellowish-brown microcrystalline powder, m.p. 149–150° (picrate of XIIb).

Anal. Calcd. for $C_{19}H_{18}$:$C_6H_3O_7N_3$: C, 63.15; H, 4.4; N, 8.8. Found: C, 62.79; H, 3.88; N, 8.78.

The oily hydrocarbon (XIIb), obtained by passing the benzene solution of this picrate through an alumina layer, was dried at a reduced pressure; λ_{max}^{EtOH} 266 mμ (Fig. 1).

Anal. Calcd. for $C_{19}H_{18}$: C, 92.65; H, 7.35. Found: C, 92.87; H, 7.00.

The crude XIIc was dissolved in 20 cc. of benzene, passed through a column containing 30 g. of alumina, and the column was washed with 80 cc. of benzene. The effluent was fractionated into 20-cc. portions, and the crystals obtained from fractions 1–3 were recrystallized from alcohol to colorless needles (XIIc), m.p. 210–212°.

Anal. Calcd. for $C_{19}H_{18}O$: C, 87.0; H, 6.8. Found: C, 86.57; H, 6.92.

HAKATA, FUKUOKA, JAPAN

[CONTRIBUTION FROM THE EASTERN UTILIZATION RESEARCH BRANCH, AGRICULTURAL RESEARCH SERVICE, U. S. DEPARTMENT OF AGRICULTURE]

Steroidal Sapogenins. XIV. Hydrolysis of 5α,22a-Spirostane Glycosides by Plant Enzymes[1-3]

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RECEIVED FEBRUARY 1, 1954

Enzymes present in leaves of Agaves and Yuccas containing saponins with 5α,22a-spirostane aglycones will cleave many saponins to their aglycones and component sugars or polysaccharides. These enzyme systems are called saponases. Saponases are active in the present of 10% ethanol. Higher concentrations of ethanol or 10% butanol inhibit saponase activity. Tigogenin, hecogenin, gitogenin and manogenin are typical aglycones recovered from the hydrolysates. The aglycones or sapogenins produced by the action of saponases are identical with sapogenins obtained from the same source by hydrolysis with 2–4 *N* HCl.

A preliminary communication[4] from this Laboratory reported the hydrolysis of steroidal saponins by enzymes that occur in the same plants as the saponins. In presenting a more detailed account of the occurrence and properties of these enzymes, the researches reported will be restricted to a discussion of the hydrolysis of steroidal saponins by enzymes present in plants containing saponins with 5α,22a-spirostane aglycones; *e.g.*, tigogenin, hecogenin, gitogenin and manogenin. The hydrolysis of saponins having aglycones of different configuration will be discussed in other papers.

Partial enzymatic hydrolysis of cardiac glycosides to monoglycosides has been known for 20 years.[5] More recently, plant and mold enzymes have been discovered which can split the carbohydrate–steroid linkage[6-8] in the cardiac series. Except for a rather inconclusive note by Canham and Warren,[9] however, neither type of enzymatic hydrolysis has been demonstrated previously on steroidal saponins.

We have found that plant enzymes can cleave 5α,22a-spirostane glycosides to the steroidal sapogenin and component sugars. We will call such enzyme systems saponases, reserving the term hemisaponases for enzymes that leave one or more sugars attached to the steroid nucleus.

Saponases have been found in the leaf tissues of a number of Agave species and also in *Yucca gloriosa*. As shown in Table I, the saponin substrates prepared from these plants all yielded 5α,22a-spirostanes. Since extensive sapogenin surveys[1,10] have failed to show the presence of free sapogenins in freshly harvested plants, apparently *in vivo* the enzymes and substrate are kept rigidly separate.

That saponases do exist is easily demonstrable. By grinding fresh leaf tissue of the species given in Table I, and extracting the ground material with cold water, a solution is obtained that becomes turbid on standing. Part of the turbidity is due to the water-insoluble sapogenins formed by hydrolysis of the water-soluble saponins. Although research now in progress may modify the present statements, the best experimental conditions for saponase activity seem to be a temperature of 30–37° for 48–96 hr. with the *p*H about 5.3. The crude sludge thus obtained contains 25–40% sapogenins. As described previously,[11] the sapogenins

(1) Paper XIII, M. E. Wall, *et al.*, AIC-363, February, 1954.

(2) Presented in part at the Philadelphia Meeting-in-miniature of the American Chemical Society, January 29, 1953. Not copyrighted.

(3) Previous work at this Laboratory has shown that steroidal saponins can be regarded as spirostane-3 glycosides. E. S. Rothman, M. E. Wall and C. R. Eddy, THIS JOURNAL, **74**, 4013 (1952).

(4) M. M. Krider and M. E. Wall, *ibid.*, **74**, 3201 (1952).

(5) A. Stoll, *et al.*, *Helv. Chim. Acta*, **34**, 1933 (1951), and W. A. Jacobs, *Physiol. Rev.*, **13**, 222 (1933).

(6) A. Stoll, A. Pereira and J. Renz, *Helv. Chim. Acta*, **32**, 293 (1949).

(7) A. Stoll, J. Renz and A. Brack, *ibid.*, **34**, 397 (1951).

(8) H. Huber, *et al.*, *ibid.*, **34**, 46 (1951).

(9) P. A. S. Canham and F. L. Warren, *J. S. African Chem. Inst.*, [N. S.], **3**, 9 (1950).

(10) R. E. Marker, *et al.*, THIS JOURNAL, **69**, 2167 (1947).

(11) M. E. Wall, M. M. Krider, E. S. Rothman and C. R. Eddy, *J. Biol. Chem.*, **198**, 533 (1952).