evaporated. The first section (186 mg.) was covered with benzene and filtered to remove crystals of vanillic acid. The benzene solution upon rechromatographing yielded a yellow product melting at 110–120° which, upon further chromatography, yielded only vanillic acid melting at 209–210°. The second section (1.15 g.) was recrystallized from water to yield vanillic acid melting at 209–210°. The third section (390 mg.) was recrystallized from petroleum ether (b.p. 65–110°) to give acetovanillone melting at 110–111° and not depressing a mixed melting point with authentic acetovanillone. Section four, weighing 106 mg. and having a crude melting point of 109–128°, was boiled with petroleum ether and filtered. Recrystallization of the precipitate from water gave fine white crystals melting at 193–194°. The compound has not been identified. Concentration and cooling of the petroleum ether yielded needles of pure acetovanillone melting at 111–112°. The effluent upon evaporation and recrystallization gave more of the 193–194° melting compound.

The aqueous solution, after the above-described benzene extraction, was then extracted with ether, and the ether was dried and distilled to yield 7.12 g. of residue. The residue was boiled with an excess of 10:1 benzene–ethanol and filtered to leave 2 g. of crystals which were recrystallized from water to yield pure 5-carboxyvanillin melting at 256–257° and not depressing the melting point of a mixture with authentic 5-carboxyvanillin.[9] The benzene–ethanol filtrate was chromatographed on a large column of acid-washed Magnesol and developed with a large excess of 20:1 benzene–ethanol. Elution of the Magnesol yielded only a product which upon repeated recrystallization from water gave light brown crystals melting at 222–226° and giving a purple color with ferric chloride. The product has not been identified. The effluent, upon evaporation and boiling with benzene, gave vanillic acid as a precipitate (3.7 g.) and a filtrate which upon chromatography yielded crystals, melting at 113–118°, which have not been identified.

Other fractions shown in Fig. 1, except fractions I, II and III, were analyzed in an analogous manner.

(9) I. A. Pearl and D. L. Beyer, THIS JOURNAL, **74**, 4263 (1952).

Representative Fractionation by Paper Partition and Cellulose Column Chromatography. Analysis of Fraction I.—Fraction I (10 g.) was dissolved in benzene and chromatographed on acid-washed Magnesol (70 mm. in diameter by 330 mm. in length), and developed with 1300 cc. of 20:1 petroleum ether (b.p. 65–110°)–ethanol. Ferric chloride, 2,4-dinitrophenylhydrazine and alkaline permanganate streak reagents indicated four bands. The column was cut into four sections, and each section was eluted with acetone. The acetone eluates were evaporated to dryness. The four residues were dissolved in acetone and spotted on Whatman No. 1 filter paper in quadruplicate, developed in a descending system with butanol saturated with 2% aqueous ammonia, and the chromatograms sprayed separately with three reagents as described earlier.[1] Results were: section 1, trace of vanillic acid at R_f 0.10; section 2, strong vanillin at R_f 0.48, and a strong spot at R_f 0.78; section 3, strong vanillin at R_f 0.48, spot at R_f 0.65 and at R_f 0.87; and section 4, light vanillin spot at R_f 0.48, acetovanillone at R_f 0.60, and spot at R_f 0.87.

All spots were eluted from the unsprayed chromatogram as described earlier.[1] Rechromatographing of the eluted R_f 0.78 spot of section 2 and of the R_f 0.65 spot of section 3 under the same conditions gave only spots of pure acetovanillone at R_f 0.60. The R_f 0.87 spots of sections 3 and 4 on rechromatographing gave spots at only R_f 0.87. All eluates were identified by comparison with authentic samples (mixed melting points and ultraviolet absorption spectra).

Larger amounts (3.0 g.) of sections 3 and 4 were also chromatographed on a large column of cellulose powder and developed with butanol saturated with 2% aqueous ammonia, as described earlier.[1] Samples of the effluent were collected in an automatic fraction collector and individually spotted on paper. In this manner the same materials were isolated in a pure state in large amounts. It is interesting to note that the materials came off the column in the following order: R_f 0.87, vanillin at R_f 0.48, acetovanillone at R_f 0.60, and vanillin at R_f 0.48.

APPLETON, WISCONSIN

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF HARVARD UNIVERSITY]

Cholesteryl Naphthylcarbonates[1]

BY KWAN-CHUNG TSOU

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Cholesteryl and cholestanyl 2-naphthylcarbonate and cholesteryl 1-napthylcarbonate were prepared as chromogenic substrates for the study of cholesterol esterase. Preliminary data on the enzymatic hydrolysis of these substrates are given. Pyrolysis of cholesteryl 2-naphthylcarbonate yields mainly 2-naphthol, carbon dioxide and $\Delta^{3,5}$-cholestadiene, together with small amounts of a hitherto undescribed $\Delta^{2,5}$-cholestadiene, cholesteryl 2-naphthyl ether, 2,2′-dinaphthylcarbonate, and impure dicholesteryl ether. Characterization and properties of the new cholestadiene are described.

Since cholesterol and esters of cholesterol[2] are important constituents of living cells and tissues and human blood, enzymes involved in formation and hydrolysis of esters of cholesterol are of interest, and methods for demonstrating such enzymatic activity would be important in studying the role of cholesterol in health and disease. Esters of cholesterol with naphthylcarbonic acids are particularly suitable as substrates, since on enzymatic hydrolysis they should yield first naphthoxyformic acids and then naphthols which can be measured colorimetrically after coupling with a suitable diazonium salt.

Cholesteryl 1- and 2-naphthylcarbonate were prepared by the reaction of cholesterol with the corresponding naphthylchlorocarbonate in pyridine with protection against moisture. Cholestanyl 2-naphthylcarbonate was prepared similarly. Enzymatic studies on these substrates[3] were carried out in tissue homogenates at both *p*H 7.4 and 5.4.[4] Hydrolysis is very slow and is not complete even after 20 hours incubation, owing, at least in part, to the poor solubility of the esters. Klein[4] also noted slow hydrolysis of synthetic cholesteryl esters when in colloidal suspension.

Since Reichstein and co-workers[5] have found that

(1) This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, Department of Health, Education and Welfare, (C312-C8).

(2) L. F. Fieser, *Science*, **119**, 710 (1953).

(3) Performed by Drs. H. A. Ravin and A. M. Seligman, Beth Israel Hospital, Boston, Mass.

(4) These two *p*H's were chosen because Klein (*Z. physiol. Chem.*, **254**, 1 (1938)) found that mammals have at least two cholesterol esterases. One acts in acid solution (*p*H 5.3) and is present in liver, spleen, kidney and other tissues; the other is active at a neutral *p*H and is apparently limited to the pancreas.

(5) J. von Euw, A. Lardon and T. Reichstein, *Helv. Chim. Acta*, **27**, 821 (1944); A. Lardon and T. Reichstein, *ibid.*, **28**, 1420 (1945).

pyrolysis of esters to give the corresponding unsaturated compound is facilitated by an increase in the size of the acid moiety, pyrolysis of cholesteryl 2-naphthylcarbonate has been examined in the present study. By analogy with the products of pyrolysis of ethyl 2-naphthylcarbonate,[6] the cholesteryl ester would be expected to yield mainly 2-naphthol, carbon dioxide and $\Delta^{3,5}$-cholestadiene, known to be the predominant pyrolysis product of other cholesteryl esters.[7] A kinetic study of pyrolysis of cholesteryl carbonates has been reported recently by O'Connor and Nace,[8] who found that the reaction is essentially unimolecular, like that of xanthates. Pyrolysis of the naphthylcarbonate proceeds rapidly at 280°, with production of 2-naphthol and carbon dioxide, as expected. The diene fraction, however, had a specific rotation of −100°, a somewhat lower value than that of the purest sample of $\Delta^{3,5}$-cholestadiene (−123°[7]). The contaminant was eventually isolated by chromatography and characterized as an isomeric, unknown diene, m.p. 73–74°, α_D −24.9°. The double bonds are not conjugated, since the diene is transparent in the ultraviolet region from 236–400 mμ; strong end absorption with a maximum at 210 mμ (ϵ 11200) is indicative of isolated double bonds. It reacts with tetranitromethane to give an orange color that persists for 20 minutes; under similar conditions the colors obtained by $\Delta^{3,5}$- and $\Delta^{4,6}$-cholestadiene fade within 5 minutes. Attempted isomerization of the diene to a conjugated diene with hydrogen chloride leads to a chloro derivative, as yet not characterized. Partial isomerization (judged by the absorption maxima) occurs when the diene is heated at 320–340° at 40 mm. in a sealed tube for 6 hours. Catalytic hydrogenation furnishes cholestane. The most reasonable structure for this new diene is that of $\Delta^{2,5}$-cholestadiene. Indeed, pyrolysis of cholestanyl esters leads to a mixture of Δ^{2}- and Δ^{3}-cholestene.[9]

In addition to these main products, two other by-products were characterized. A very small amount of cholesteryl 2-naphthyl ether, m.p. 129.5–130°, was isolated, characterized further by analysis and infrared spectrum. The optical rotation data rule out the possibility of Δ^{4}-cholestenol 2-naphthyl ether (dextrorotatory). Isolation of the ether suggests that, as in the pyrolysis of ethyl 2-naphthylcarbonate, homolytic cleavage occurs to a slight extent. 2,2′-Dinaphthylcarbonate was another by-product, identified by infrared spectrum and a mixed melting point with an authentic sample.[10] This product could arise from an intermolecular reaction of two moles of the ester. Furthermore, formation of this carbonate should be accompanied by dicholesteryl ether which was in fact isolated, but only in crude form.

(6) K. C. Tsou and A. M. Seligman, THIS JOURNAL, **76**, 3704 (1954).

(7) L. F. Fieser and M. Fieser, "Natural Products Related to Phenanthrene," Reinhold Publishing Corp., New York, N. Y., 1949, p. 249.

(8) G. L. O'Connor and H. R. Nace, THIS JOURNAL, **74**, 5454 (1952); **75**, 2118 (1953).

(9) D. H. R. Barton and W. J. Rosenfelder, *J. Chem. Soc.*, 1048 (1951).

(10) G. Wolf and A. M. Seligman, THIS JOURNAL, **73**, 2080 (1951).

Experimental[11,12]

Cholesteryl 2-Naphthylcarbonate.—To a solution of 2.0 g. of cholesterol in 10 cc. of dry pyridine was added 1.0 g. of 2-naphthyl chlorocarbonate[13] in 10 cc. of dry ether. The resulting mixture was warmed for 10 minutes on a steam-bath, allowed to cool to room temperature with stirring for 2 hours, and then poured into 100 cc. of ice-water. The crystalline product was collected, washed with water, then with 95% ethanol, and recrystallized from chloroform and 95% ethanol to give 2.4 g. (83%) of white crystals, m.p. 172–173°. Further purification by chromatography over acid-washed alumina (Merck and Co., Rahway, N.J.) and elution with benzene raised the melting point to 177.5–178°, α_D −29.0° (*c* 1.93, chf.); λ^{chf} 5.70, 6.10, 6.20, 8.0 μ.

Anal. Calcd. for $C_{38}H_{52}O_3$ (556.80): C, 81.96; H, 9.41. Found: C, 81.64; H, 9.53.

Cholestanyl 2-Naphthylcarbonate.—This was prepared in the same way from cholestanol in 80% yield, recrystallized from benzene–ethanol, m.p. 145–145.5°. Chromatographic purification over acid-washed alumina afforded a pure sample, m.p. 146–146.5°, α_D +10.3° (*c* 1.74, chf.), λ^{chf} 5.70, 6.10, 6.20, 7.92 μ.

Anal. Calcd. for $C_{38}H_{54}O_3$ (558.81): C, 81.67; H, 9.74. Found: C, 81.66; H, 9.99.

1-Naphthyl Chlorocarbonate.—This was prepared by a modification of the procedure of Oesper, Broker and Cook.[14] To a dry benzene solution of phosgene (5 g., 0.05 mole) was added 6.5 g. of dry quinoline and 7.2 g. of 1-naphthol. The reaction mixture was allowed to stand at room temperature overnight, and the precipitated quinoline hydrochloride was removed by filtration. The filtrate was washed quickly with the following ice-cold solutions: 1% hydrochloric acid, tap water, 1% sodium hydroxide and then dried over anhydrous sodium sulfate. The solution was evaporated under reduced pressure and distilled to give 4.9 g. (47.5%) of the acid chloride as a light yellow oil, b.p. 117° (1 mm.) (lit.[14] b.p. 132° (5 mm.)); λ^{chf} 5.62, 6.10, 6.22, 6.32, 7.20, 9.0 μ. Unlike the 2-naphthyl chlorocarbonate, which was fairly stable in ice-water without extensive hydrolysis, this acid chloride is sensitive to moisture and discolors within 2 days.

Cholesteryl 1-Naphthylcarbonate.—To a dry pyridine solution (10 cc.) of cholesterol (2.0 g., dried over phosphorus pentoxide) was added by drops 1.0 g. of the above acid chloride. An orange addition product was formed, which gradually turned yellow. The reaction mixture was warmed gently with exclusion of moisture till all solids were dissolved. The ester crystallized when cooled; methanol was added to complete the precipitation. The product was collected and washed with methanol; colorless small crystals, 1.7 g. (59%), m.p. 171–174°. The crude product was purified by chromatography to give 1.4 g. of pure ester, m.p. 177–178°, α_D −19.2° (*c* 1.83, chf.); λ^{chf} 5.70, 6.22, 7.90, 8.07, 8.18, 10.60 μ.

Anal. Calcd. for $C_{38}H_{52}O_3$ (556.80): C, 81.96; H, 9.41. Found: C, 81.54; H, 9.63.

Pyrolysis of Cholesteryl 2-Naphthylcarbonate.—A 2.11-g. sample of pure cholesteryl 2-naphthylcarbonate was refluxed at 280° under 32 mm. in a Woods metal-bath for 2 hours. The colorless oil (1.39 g.) crystallized slowly and 2-naphthol sublimed into the condenser and above the neck of the flask (0.55 g., m.p. 122.5–123°). Net loss of weight due to carbon dioxide was 0.17 g. (quantitative). The oily residue was chromatographed over 43 g. of acid-washed alumina and eluted as usual with 100-cc. portions of petroleum ether and then 5, 10 and 25% benzene in petroleum-ether, benzene and ether. Fractions eluted by petroleum-ether when combined yielded 1.36 g. (97%) of impure $\Delta^{3,5}$-cholestadiene, m.p. 73.5–76°, α_D −100.2° (*c* 1.54, chf.). Recrystallization twice from ethyl acetate–methanol produced needles, m.p. 77–79°, α_D −100.3° (*c* 1.35, chf.), α_D −102.5° (*c* 1.42 in 5% ethanol–chloroform containing 1%

(11) All melting points are corrected.

(12) Microanalyses by Mrs. Shirley Golden of this Laboratory and by Dr. S. M. Nagy and Associates, Microchemical Laboratory, Massachusetts Institute of Technology, Cambridge.

(13) H. A. Ravin, K. C. Tsou and A. M. Seligman, *J. Biol. Chem.*, **191**, 843 (1951).

(14) R. E. Oesper, W. Broker and W. A. Cook, THIS JOURNAL, **47**, 2609 (1925).

hydrogen chloride, no change after 3 hours[15]); λ_{max}^{ether} 235 mμ (17500), no max. at 260 mμ.

From the 5% benzene fractions, a trace amount of oil was crystallized by addition of alcohol. This crude sample melted at about 206–207° after initial sintering around 170°; its infrared spectrum (micro cell) showed a diffused band at 8.9–9.1 μ.[16]

The oil from the 25% benzene fraction yielded cholesteryl 2-naphthyl ether which was recrystallized from ethyl acetate–ethanol; 18 mg., m.p. 129.5–130°, α_D −50.3° (*c* 0.91, chf.); $\lambda_{max}^{95\% EtOH}$ 230 mμ (69200), 255 mμ (2990), 265 mμ (4360), 274 mμ (4830), 284 mμ (3220). It does not couple with tetrazotized di-*o*-anisidine in bicarbonate solution. Its infrared spectrum was compared with that of the ethyl 2-naphthyl ether and differs from the latter only in a much stronger methylene band at 3.4 μ and a shift of the 9.60 μ band to 9.80 μ. When the naphthyl ether was dissolved in 8% bromine in glacial acetic acid and allowed to stand for 1 hour at room temperature, and the precipitated dibromide was separated by centrifuge; naphthol was absent as shown by a negative coupling reaction with tetrazotized di-*o*-anisidine in bicarbonate suspension.

Anal. Calcd. for $C_{37}H_{52}O$ (512.79): C, 86.66; H, 10.22. Found: C, 86.54; H, 10.02.

The ether fraction yielded 5 mg. of 2,2′-dinaphthylcarbonate, which upon recrystallization from ethanol melted at 170–171°. It was identified by comparison of its infrared spectrum with that of an authentic sample.[10] It gave no melting point depression on admixture with this sample.

$\Delta^{2,5}$-Cholestadiene.—Due partly to the small amount present, isolation of this diene from the above reaction product was difficult. A 10-g. sample of cholesteryl 2-naphthylcarbonate was pyrolyzed at 255° and 30 mm. under nitrogen atmosphere for 2 hours. The diene mixture (5.9 g.) isolated as above was rechromatographed over 180 g. of acid-washed alumina and eluted with petroleum–ether (b.p. 30–60°) in 50-cc. fractions. Fractions 1–4, on evaporation gave only a trace of oil; fraction 5 afforded 0.1 g. of $\Delta^{2,5}$-cholestadiene as solid which was twice recrystallized from ethanol and a few drops of ether to yield 67 mg. of small white crystals, m.p. 73–74°, α_D −24.9° (*c* 2.69 in chf.); $\lambda_{max}^{95\% EtOH}$ 210 mμ (11200), transparent from 236–400 mμ[17]; λ^{chf} no hydroxyl absorption, 3.40–3.48, 6.10, 6.80, 7.25 μ.

Anal. Calcd. for $C_{27}H_{44}$ (368.62): C, 87.97; H, 12.03. Found: C, 87.73; H, 12.10.

Microhydrogenation was carried out by the method of Clauson-Kaas and Limborg[18]; 8.553 mg. of this sample in 5 cc. of glacial acetic acid took up 0.843 cc. of hydrogen, calculated to show the presence of two double bonds. Evaporation of the hydrogenated solution gave white lustrous plates, m.p. 77–78°, identified as cholestane (no depression of melting point on admixture with an authentic sample). Tetranitromethane test of this diene in chloroform gave an orange color which did not fade in 20 minutes, whereas under identical conditions $\Delta^{3,5}$- and $\Delta^{4,6}$-cholestadiene gave a similar color which faded in 5 minutes. A chloroform solution of $\Delta^{2,5}$-cholestadiene (4 mg.) was saturated with dry hydrogen chloride and stored overnight at 0°. Upon evaporation to dryness, the residue melted at 88–92° (hot-stage); ultraviolet spectrum shows end absorption only which was much weaker than the starting material; positive Beilstein test and C–Cl band (13.10 μ) in its infrared spectrum in carbon disulfide solution; the tetranitromethane test gave only lemon-yellow color. A 4-mg. sample of $\Delta^{2,5}$-cholestadiene was sealed at 40 mm. and heated for 6 hours at 320–340° in a Woods metal-bath. The resulting oil showed strong blue fluorescence in ultraviolet light; λ_{max}^{ether} 229 mμ (6780), 234 mμ (6920), 242 mμ (4970), 261 mμ (2220).[19]

Fractions 6 and 7 gave an oily residue, 1.2 and 1.6 g., respectively. Repeated chromatography of fraction 6 on alumina in the same manner isolated another 0.1 g. of $\Delta^{2,5}$-cholestadiene, and an estimated (ultraviolet) 0.7 g. should be isolable in the following fractions; total $\Delta^{2,5}$-cholestadiene, 0.9 g. (15% of the diene mixture). From fractions 7 to 11, 3.1 g. of pure $\Delta^{3,5}$-cholestadiene was obtained, m.p. 78–79°, after three recrystallizations from acetone,[20] α_D −120.7° (*c* 2.10. chf.); $\lambda_{max}^{95\% EtOH}$ 228 mμ (20000), 235 mμ (22000), 243 mμ (14300). Fractions 12 to 15 gave a small amount of an oil which showed blue fluorescence in ultraviolet light.

Enzymatic Hydrolysis.[3]—The experimental technique in enzymatic study is the same as that given in a previous paper.[21] The period of incubation with homogenized tissue of the rat was 20 hours. For *p*H 7.4, 0.1 *M* veronal buffer was used, and for *p*H 5.4, 0.1 *M* acetate buffer. The hydrolysis of these substrates was found to be extremely slow and a typical experiment is shown in Table I.

TABLE I

RATES OF HYDROLYSIS OF 2-NAPHTHYLCARBONATES

Enzyme source	Cholesteryl *p*H 7.4	Cholesteryl *p*H 5.4	Cholestanyl *p*H 7.4	Cholestanyl *p*H 5.4
Liver	12[a]	5[a]	3[a]	1.5[a]
Kidney	6	5	4	1.7
Pancreas	7	5	5	2
Intestine	5	2.5	15	2

[a] Micrograms naphthol per mg. tissue per hour.

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CAMBRIDGE 38, MASS.

(15) This stability indicates that $\Delta^{3,4}$-cholestadiene is not present in significant amount.

(16) A comparison of this infrared spectrum with that of dicholesteryl ether (m.p. 203–204°, kindly provided by Dr. R. Stevenson of this Laboratory) indicated that they differ only in the diffused nature of this band.

(17) Ultraviolet spectra were determined with a Cary Recording Spectrophotometer, Model 11M, Applied Physics Corporation, Pasadena, Calif.

(18) N. Clauson-Kaas and F. Limborg, *Chim. Acta Scand.*, **1**, 884 (1947).

(19) Assuming these maxima are due to a mixture of $\Delta^{3,5}$- and $\Delta^{2,4}$-cholestadiene and judging by the extinction coefficients, one may estimate that 31% of the $\Delta^{3,5}$- and 29% of the $\Delta^{2,4}$-diene have been formed. Similarly, a 3-hour reaction gave only 8% of the $\Delta^{3,5}$- and 6% of the $\Delta^{2,4}$-diene.

(20) It was found that recrystallization from acetone alone gave better purification than from a mixed solvent of acetone and methanol.

(21) K. C. Tsou and A. M. Seligman, THIS JOURNAL, **74**, 3066 (1951).