cess of hydrochloric acid was added and the solution was extracted with ether. The ether was washed with dilute sodium hydroxide and with water until neutral. After drying over sodium sulfate, the solvent was removed. The reduction products were isolated by chromatography upon alumina except in the case of compounds having the dihydroxyacetone side chain. These latter products were acetylated with acetic anhydride and pyridine at room temperature. The acetates were chromatographed on a partition column using silica gel containing 40 ml. of ethanol per 100 g. of silica gel as the stationary phase and developing the column with a 1% solution of ethanol in methylene chloride. A good separation of product and starting material was obtained in all cases. In some instances, a good separation between the two C-3 isomers was achieved with the partition column; this was much more difficult with very polar substances than was the separation of the simple monohydroxy ketosteroids.

This report describes the first partial synthesis of 11β,17α-dihydroxy-3α,21-diacetoxypregnan-20-one. As with the natural material isolated from urine,[9] the melting point was not a criterion of purity. An analytical sample had a m.p. 212–222° but the rotation was constant through several recrystallizations, $[\alpha]^{24}D$ +90.8° (chloroform), $[\alpha]^{24}D$ +85.0° (acetone). The product was identical with the urinary metabolite, as was the infrared spectrum of the two products.

Acknowledgments.—We are indebted to Estella R. Katzenellenbogen and Friederike Herling for the determination and interpretation of infrared spectra, to Dr. Katzenellenbogen and Millicent Houde for help with the partition chromatography and to Max Tishler of Merck & Co., and to George Rosenkranz of Syntex S.A., Laguna Mayran, Mexico, for generous gifts of starting material.

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NEW YORK 21, N. Y.

[CONTRIBUTION FROM THE RESEARCH SERVICE, FIRST (COLUMBIA UNIVERSITY) DIVISION, GOLDWATER MEMORIAL HOSPITAL AND THE DEPARTMENTS OF BIOCHEMISTRY AND MEDICINE, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY]

Separation of the Air-oxidation Products of Cholesterol by Column Partition Chromatography

BY ERWIN H. MOSBACH,[1] MARTHA NIERENBERG AND FORREST E. KENDALL

RECEIVED DECEMBER 31, 1952

The oxygenated sterols resulting from the air-oxidation of colloidal solutions of cholesterol have been separated by column partition chromatography. Two solvent systems proved effective: aqueous methanol–cyclohexane and propylene glycol–petroleum ether. The stationary phase in each case consisted of Celite 545, partially saturated with the alcohol. It was shown that the isomeric 7-hydroxycholesterols were formed in nearly equal amounts during the air-oxidation of cholesterol sols. Cholestane-3β,5α,6β-triol which had not been identified previously as a reaction product was shown to be a minor component of air-oxidized cholesterol.

Introduction

A number of investigators have shown that the cholesterol obtained from the non-saponifiable fractions of animal tissues is accompanied by relatively minute amounts of structurally related sterols. Thus 7-ketocholesterol (or its dehydration product $\Delta^{3,5}$-cholestadiene-7-one), 7α-hydroxycholesterol, 7β-hydroxycholesterol and cholestane-3β,5α,6β-triol have been isolated from a variety of different organs such as aortas, liver, testes, spleen and blood.[2–5] Schwenk has shown recently that cholestane-3β,5α,6β-triol may be present in most samples of cholesterol prepared from animal sources.[6] It has been pointed out repeatedly, however, that with the possible exception of 7-ketocholesterol these substances may be artefacts derived from cholesterol during the isolation procedures.[2,3,7] This point of view received strong support from the work of Bergström and Wintersteiner[7,8,9] who showed that at *p*H 8 and temperatures near 85° about 65% of colloidally dispersed cholesterol was converted to 7-ketocholesterol and the isomeric 7-hydroxycholesterols within a few hours. It was found by the same authors that the oxidation took place under physiological conditions of *p*H and temperature although at a slower rate. They suggested that a conversion of cholesterol to the 7-oxygenated sterols might occur *in vivo*, and that an attack on the sensitive 7-position of the cholesterol molecule might be involved in the biological degradation of this sterol.[7]

The present study on the separation of the air-oxidation products of cholesterol had as its objective the development of procedures for an investigation of the biological fate of these sterols by radioactive tracer techniques.[10] Although the separation of weakly polar sterols has been achieved by paper chromatography,[11] it was decided to investigate the use of columns so that somewhat larger quantities of sterols, required for C^{14}-tracer studies, could be handled.

Two solvent systems proved useful for partition-chromatographic separations: 95% aqueous methanol–cyclohexane, and propylene glycol–petroleum ether (b.p. 60 to 68°). The alcoholic component, supported on Celite 545, was used as the stationary phase in both cases. It was found that the major components of air-oxidized cholesterol sols could be

(1) Fellow of the Public Health Research Institute of the City of New York, Inc., and Research Fellow, Research Service, First (Columbia University) Division, Goldwater Memorial Hospital.

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separated satisfactorily with the system propylene glycol–petroleum ether. Columns prepared with aqueous methanol–cyclohexane were employed for the separation of cholestane-3β,5α,6β-triol from the other oxidation products. This compound had not been identified previously as a constituent of air-oxidized cholesterol sols.

Experimental

Preparation of "Oxidized Cholesterol."—The air-oxidation procedure of Bergström and Wintersteiner[8] was modified slightly so that 100-mg. quantities of cholesterol could be processed conveniently and reproducibly: A solution was prepared containing 2.5 mg. of recrystallized stearic acid and 3 mg. of trisodium phosphate per ml. of 37.5% aqueous ethanol. This solution was neutralized with sodium hydroxide (phenolphthalein), and 8 ml. of this solution was diluted to about 50 ml. with water and placed in a 250-ml. cylindrical flask provided with an efficient stirrer. Sufficient turbulence was produced by the stirrer so that no air-inlet tube had to be provided. The flask was heated to 70° in a water-bath, and the cholesterol to be oxidized (about 100 mg.) was added in 10 ml. of boiling absolute ethanol. Stirring was continued for 6 hours, and the temperature was raised gradually to 85° to evaporate most of the ethanol. Water was added during the oxidation as required to keep the volume approximately constant. The sterols and stearic acid were extracted with ether from the acidified (HCl) reaction mixture. The sterols were separated from the stearic acid by extraction of the ether solution with aqueous 5% KOH. Yields of total sterols ranged from 90 to 97% based on cholesterol.

Apparatus.—The partition columns and their method of preparation have been described.[12] The stationary phases (95% aqueous methanol or propylene glycol) were equilibrated with the corresponding mobile phases (cyclohexane or petroleum ether, 60–68°) before use. The system methanol–cyclohexane is sensitive to temperature changes and may assume a cloudy appearance but this does not seem to affect the operation of the columns. Sterol samples were placed on the column dissolved in a minimum volume (1 to 3 ml.) of hot mobile phase or incorporated into Celite together with a small amount of the stationary phase.[12] An automatic fraction collector was used to collect serial fractions of 3 to 7 ml. each. Flow rates were about 0.5 ml. per minute but did not appear to have a marked effect on the completeness of separation. The capacity of the 45 cm. long methanol–cyclohexane columns (retention volume 20 ml.) was about 50 mg. of sterols, while propylene glycol–petroleum ether columns of the same length had about double this capacity. Larger columns, 3.14 sq. cm. × 30 cm., were found to be useful for the isolation of cholestane-3β,5α,6β-triol.

Test Substances.—Cholesterol (m.p. 148–149°) to be oxidized was recrystallized 3 or more times from ethanol and contained about 3% of dihydrocholesterol. The known sterols were synthesized by published procedures, except 7α-hydroxycholesterol and 7β-hydroxycholesterol which were prepared by reduction of 7-ketocholesterol with sodium borohydride.[13] Wintersteiner's compound A was prepared by treating 7β-hydroxycholesterol with ethanolic acetic acid.[8,14] The 7-oxygenated sterols were assayed as described by Bergström and Wintersteiner,[8] cholesterol was determined by the Liebermann–Burchard reaction[15] and gravimetrically by digitonin precipitation, dihydrocholesterol by the method of Schönheimer[16]; the other sterols were assayed gravimetrically.

Cholestane-3β,5α,6β-triol.—The unknown substance obtained from some preparations of air-oxidized cholesterol on methanol–cyclohexane chromatograms (see Results and Discussion) gave no color reaction with Lifschütz or Liebermann–Burchard reagents, did not absorb in the ultraviolet above 220 mμ and formed a digitonide slowly at room temperature.[17] Recrystallized from aqueous methanol, then from benzene, it melted at 235–238° (not corrected) and showed no melting point depression with a known sample of cholestane-3β,5α,6β-triol.[18] The diacetate melted at 163–166° (not corrected) and showed no melting point depression with an authentic sample of cholestane-3β,5α,6β-triol-3,6-diacetate.[2]

The elution band of the sterol isolated from the oxidation mixture coincided with that of the known cholestane-3β,-5α,6β-triol, but did not coincide with the elution bands of either cholestane-3β,5α,6α-triol[19] or cholestane-3β,5α-diol-6-one.[18] The 6-ketone was examined since it did not give a melting point depression with cholestane-3β,5α,6β-triol.[6]

Anal. Calcd. for $C_{27}H_{48}O_3$: C, 77.1; H, 11.5. Found: C, 76.6; H, 11.2.

Results and Discussion

Figure 1 shows a composite diagram of the position of various sterols on a methanol–cyclohexane chromatogram. The diagram indicates that cholesterol and 7-ketocholesterol are separated only partially, while both of these sterols are separated from the 7-hydroxycholesterols. The resolution of the latter compounds is likewise quite incomplete with this solvent system. However, when some preparations of "oxidized cholesterol" were chromatographed with this solvent system a fifth compound appeared after 7-hydroxycholesterol. This compound was identified as cholestane-3β,5α,6β-triol as described in the Experimental section. It should be pointed out that the cholestanetriol could not be detected in all preparations of "oxidized cholesterol"; the reason for this is not certain, since a number of factors may influence the course of the reaction.[9]

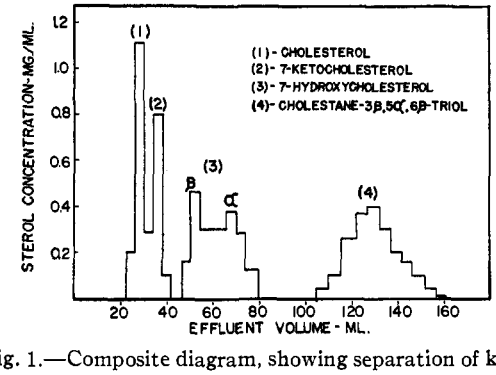

Fig. 1.—Composite diagram, showing separation of known sterols: 0.79 sq. cm. × 45 cm. Celite column. Solvent system: cyclohexane–95% aqueous methanol; 5 mg. each of cholesterol, 7-ketocholesterol, 7α-hydroxycholesterol, 7β-hydroxycholesterol, 10 mg. of cholestane-3β,5α,6β-triol.

Since the system methanol–cyclohexane did not afford very satisfactory separations, other solvent combinations were investigated in order to achieve better resolutions. Figure 2 shows a partition

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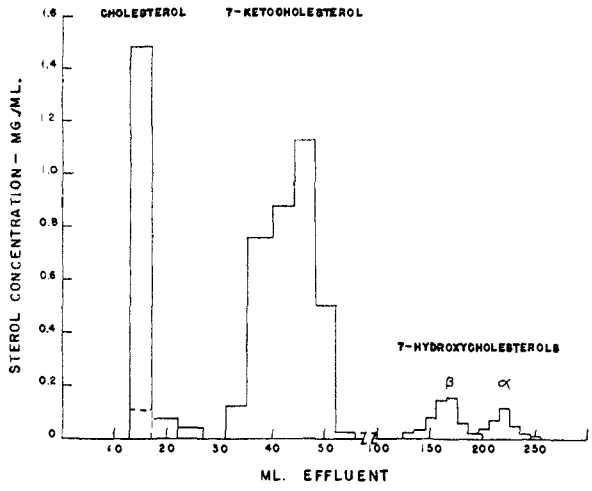

Fig. 2.—Separation of 32.5 mg. of "oxidized cholesterol" by column partition chromatography; 0.79 × 30 cm. Celite column; solvent system, petroleum ether (60–68°)–propylene glycol.

chromatogram obtained with propylene glycol as the stationary phase and petroleum ether (60–68°) as the mobile phase. The diagram demonstrates the fraction of 32.5 mg. of "oxidized cholesterol" on a 30-cm. column. With this solvent system cholesterol and 7-ketocholesterol could be separated completely, while the resolution of the isomeric 7-hydroxycholesterols was almost complete. The latter were separated more completely on columns 45 cm. in length. Cholestane-3β,5α,6β-triol was eluted with great difficulty from this type of column, and was usually determined with the methanol–cyclohexane system described above. It is interesting to note that columns prepared with propylene glycol as the stationary phase could be reused repeatedly, but care had to be taken to "regenerate" the column after each use by washing with adequate amounts of petroleum ether.

The dotted line at the bottom of the cholesterol peak in Fig. 2 indicates that this fraction contained a mixture of sterols. More than 90% of the fraction was cholesterol as determined colorimetrically[15] and by digitonin precipitation. A major impurity was dihydrocholesterol which probably was carried unchanged through the oxidation and extraction procedures. The Lifschütz reaction showed the presence of small amounts of chromogens, probably Wintersteiner's compound A.[5,14] Examination of the cholesterol band in the ultraviolet disclosed two peaks, one at 230 mμ, the other at 270 mμ (in cyclohexane), suggesting the presence of Δ^4-cholestene-3-one and $\Delta^{3,5}$-cholestadiene-7-one. The latter probably was derived from 7-ketocholesterol.[7]

Table I shows yield data for two typical oxidation experiments as determined by partition chromatography in conjunction with ultraviolet spectrophotometry, colorimetry and gravimetric determinations. The values for the major oxidation products (upper part of Table I) are in good agreement with the published data.[9] The chromatographic procedure used in this investigation made it possible to demonstrate that the 7-hydroxycholesterols were formed in approximately equal amounts, with the β-isomer predominating slightly in most experiments. It was found that there were considerable variations in yields of the major products from experiment to experiment, although the oxidations were carried out under apparently identical conditions. It has been pointed out that these differences may be due to the presence of catalysts or the state of colloidal dispersion.[9] However, in every case 90 to 95% of the oxidized mixture could be accounted for on a weight basis. Three minor reaction products have been included in Table I (together with dihydrocholesterol) for sake of completeness. It has been mentioned above that the identification of these substances must be considered highly tentative.

TABLE I

COMPOSITION OF TYPICAL "OXIDIZED CHOLESTEROL" PREPARATIONS

Compound	Compound in mixture, % Expt. I	Expt. II
Cholesterol	20.3	20.8
7-Ketocholesterol	41.1	29.7
7α-Hydroxycholesterol	10.4	11.1
7β-Hydroxycholesterol	13.9	11.8
Cholestane-3β,5α,6β-triol[a]	7.1	2.8
Dihydrocholesterol	2.9	2.9
$\Delta^{3,5}$-Cholestadiene-7-one	0.3	0.5
Δ^4-Cholestene-3-one	0.5	1.5
Compound A[b]	0.3	1.0
Total	96.8	92.1

[a] This compound was found in about one-half of the preparations of "oxidized cholesterol." [b] Considered to be Δ^6-cholestenediol-3β,5 by Bergström and Wintersteiner, but now identified as the 7-ethyl ether of 7β-hydroxycholesterol.[15]

Recovery experiments carried out with known sterols indicated that recoveries from 30 cm. columns varied from 91.2% for 7β-hydroxycholesterol to 97.7% for 7-ketocholesterol, with column loads from 0.1 to 50 mg. Since the recoveries did not attain the theoretical values, this fact may serve to explain in part why the yields listed in Table I failed to account for a higher percentage of the oxidized mixture.

Acknowledgments.—This work was supported in part by grants from the National Heart Institute, The Public Health Research Institute of the City of New York, Inc., and the Albert and Mary Lasker Foundation.

WELFARE ISLAND, NEW YORK 17, N. Y.