Autoxidation Products from Cholesterol[1]

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Abstract

Thin-layer chromatography and gas-liquid chromatography were used to separate and identify products from the autoxidation of cholesterol. Both cholesterol-α-epoxide and 1,4-cholestadien-3-one were found. In addition, several other compounds, identified also by previous workers, were shown to be present. Autoxidation in bulk was quite slow at 82C, requiring several weeks for development of detectable quantities of decomposition products. The reaction could be accelerated by irradiation with ultraviolet light or by heating the sterol above its melting point.

Introduction

VARIOUS WORKERS have reported that cholesterol, or its oxidation products, produce tumors in test animals when administered by subcutaneous injection (1–8,14). Some even suspect highly purified cholesterol as being carcinogenic (4). In this study, however, the interest lies in the stability of cholesterol to air oxidation with emphasis on the rate of peroxide formaton and the nature of the products formed.

The literature on cholesterol autoxidation has been summarized recently by Bergstrom and Samuelson (9). Both 7α- and 7β-hydroxy- and 7-keto cholesterol have been identified in the products of photooxidation and from oxidation in aqueous colloidal solution (10,11). Also cholestan-3β, 5α, 6β-triol was found using either method of oxidation. Other products definitely identified include cholestan-3β, 5α-diol-6-one, 3,5-cholestadien-7-one, 4-cholesten-3-one, and 4-cholesten-3β, 6-diol (12). Heating cholesterol above its melting point, in the dark, had an effect similar to that of irradiation. Norcia (13) reported that solid cholesterol was relatively stable to air oxidation, but that when dissolved in films of lipids, it is readily subject to oxidative attack.

The objective of the present work was to determine the rate and course of autoxidation of cholesterol in bulk under relatively mild conditions. Purified cholesterol was held in air at 82C, and the oxidation rate was determined by measuring peroxide levels. At intervals, samples were taken for fractionation by thin-layer chromatography (TLC) and gas-liquid chromatography (GLC). In addition, samples which had been aged for prolonged periods at ambient temperature in air were analyzed.

Materials and Methods

Samples were chromatographed on Silica Gel G plates (20 × 20 cm, 250 μ) from Analtech, Inc., Wilmington, Del. These were usually activated 1 hr at 110C prior to use. The solvent was a mixture of 35/65/2—petroleum ether (bp 20–40C)/ethyl ether/acetic acid. Spots were visualized by spraying with 50% sulfuric acid and heating at 135C. Heating for 3–5 min at this temperature gave colored spots; these are described in Table III. More prolonged heating gave additional charring with the result that the various ketones could be located more easily.

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TABLE I

Analysis of Aged Cholesterol Samples

	mp (°C)[a]	25°_{D}[b]	PV(meq/kg)[c]
C. P. Cholesterol	149–151	−39.5°	20
Sample A	95–110	−32.0°	700
Sample B	100–112	−17.0°	720
Impurity from B		−12.9°	900

	NMR[d]
C. P. Cholesterol	5.2–5.6 PPM (=) 2.20 PPM (H)
Sample A	5.2–5.6 PPM—small 2.20 PPM—small
Impurity from B	5.2–5.6 PPM—missing 2.20 PPM—V. small

[a] Fisher-Johns Melting Point Block.
[b] 1% in $CHCl_3$.
[c] AOCS Official Method Cd 8-53.
[d] Relative to tetramethylsilane at 0.0 ppm.

For gas chromatography an F & M 810 Chromatograph with flame ionization detector was used. The column was 6 ft × 1/8 in. of 3% QF-1 on 100/120 Gas-Chrom Q. Trimethylsilyl (TMS) ethers were prepared from the hydroxyl-containing compounds by using the method of Ahrens et al. (15).

USP cholesterol (mp 148–149C) was obtained from the Fisher Scientific Co. Dihydrocholesterol, cholestan-3β, 5α, 6β-triol, cholesterol-α-epoxide, cholestan-3-one, 4-cholesten-3-one, 4,6-cholestadien-3-one, 7-ketocholesterol, and 1,4-cholestadien-3-one were all from Mann Research Laboratories. From Aldrich Chemical Company were obtained 7-dehydrocholesterol, 3,5-cholestadien-7-one, and 5-cholesten-3-one. A mixture of 7α- and 7-β-hydroxycholesterols was prepared by reduction of the 7-keto compound with lithium aluminum hydride (16).

Two samples of commercial cholesterol had been held at ambient temperature on the shelf in amber-colored bottles for several years. Sample A was from the Pfanstiehl Chemical Co. and Sample B was from the Paragon Testing Laboratories. These were included in the analyses, to serve as a contrast to the samples oxidized deliberately, over a relatively short period of time.

Results and Discussion

The samples of cholesterol which had been aged at ambient temperature for several years (A and B) were analyzed as outlined in Table I. Melting points and optical rotations indicate that these samples contain substantial quantities of impurities. Peroxide values show that they have become significantly autoxidized during storage.

The impurity from Sample B was concentrated in the filtrate of methanol recrystallization. It was an orange, gummy, highly peroxidized material. Relative to recrystallized USP cholesterol, these com-

TABLE II

Heating of Cholesterol at 82C

Weeks	PV (meq/kg)	% Oxirane
0	12	0.0
1	26	0.0
2	41	—
3	45	0.017
4	55	—
5	47	0.025
6	58	0.020
7	63	0.017
8	59	0.020
9	50	0.013

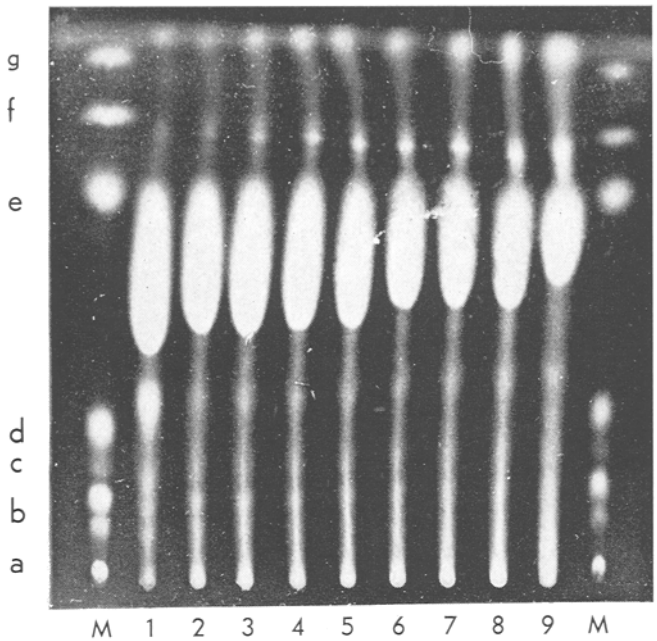

FIG. 1. TLC of 82C. Heated cholesterol (1–9 weeks). *M* is the known sterol mixture: (*a*) cholestan-3β, 5α, 6β-triol, (*b*) 7α and 7β-hydroxycholesterol, (*c*) 7-keto cholesterol, (*d*) cholesterol-α-epoxide, (*e*) cholesterol, (*f*) 4-cholesten-3-one, (*g*) 3,5-cholestadien-7-one.

mercial samples show an absence of unsaturation (Varian A-60 NMR Spectrometer). The infrared spectrum (nujol mull) of the recrystallized USP sample had a very small carbonyl peak whereas Sample A had a large carbonyl band at 1705 cm^{-1} with a shoulder at 1670 cm^{-1}. Sample B showed a large, broad carbonyl band at 1660–1710 cm^{-1}. Dobriner (17) indicates carbon-oxygen double bond stretching at 1703–1710 cm^{-1} for steroid carboxyl, 1680–1684 cm^{-1} for 1,3-dienone, and 1663–1666 cm^{-1} for 1,4-dien-3-one.

Since the infrared spectra indicated carboxyl groups, the impurity from Sample B was titrated in neutralized alcohol with sodium hydroxide. A 0.6 g sample consumed 4.0 ml of 0.1 N NaOH solution to the phenolphthalein endpoint. Sample A was also acidic in solution. Presumably a significant degree of oxidative ring cleavage had occurred during prolonged storage.

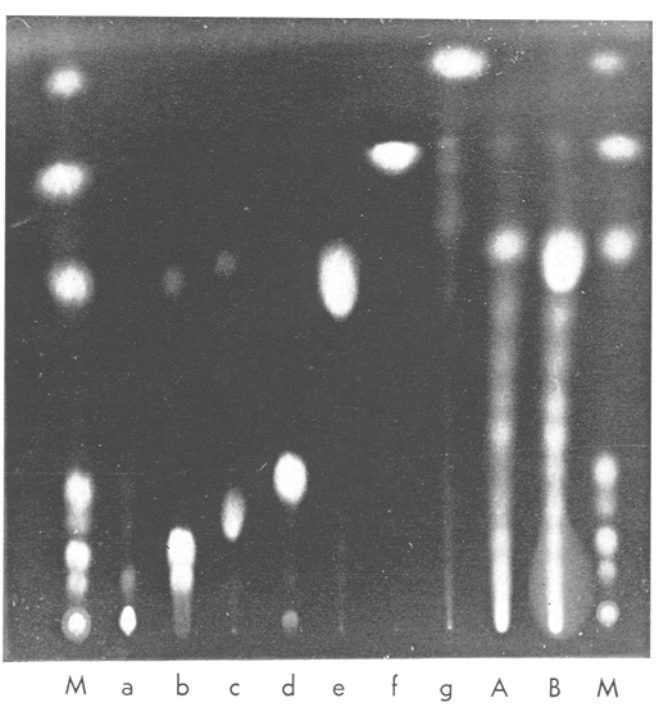

FIG. 2. TLC of known sterols. NIH (*A*) and paragon sample impurity (*B*). *M* is the mixture (*a-g*) identified in Fig. 1.

Heating Experiment

A 50 g sample of purified cholesterol (mp 148–150C) was spread on aluminum foil in a forced draft oven held at 82C. Samples were taken at weekly intervals for peroxide (18) and epoxide determinations (19). Although the sample became quite yellow in color during the 9-week period, the amount of oxidation was minimal, as shown in Table II. Titration with HBr by the Durbetaki technique gave values which were difficult to reproduce because the volumes of titrant were quite small and the end-points showed some drifting. The values for oxirane content appear to reach a maximum at about 5 weeks and to decrease slowly thereafter. Attempts were made to apply the previously reported picration technique to cholesterol epoxide (20) without success. The Urbanski method (21) based upon the addition of 2,4-dichlorobenzenesulfonic acid was also unsatisfactory. The reagent reacts quantitatively with the epoxide, but with heated cholesterol it gives unreasonably high epoxide content, indicating it is not selective for oxirane.

Thin-Layer Chromatography

In Fig. 1 is shown a negative print made from a transparency indicating the separations obtained with the samples of cholesterol which had been heated at 82C from 1 to 9 weeks in air. In each case 500 μg in chloroform was spotted at the origin, developed, and sprayed with 50% sulfuric acid. Also included is a known mixture (M) of sterols (25 μg each). Starting from the origin, spot *a* is cholestan-3β,

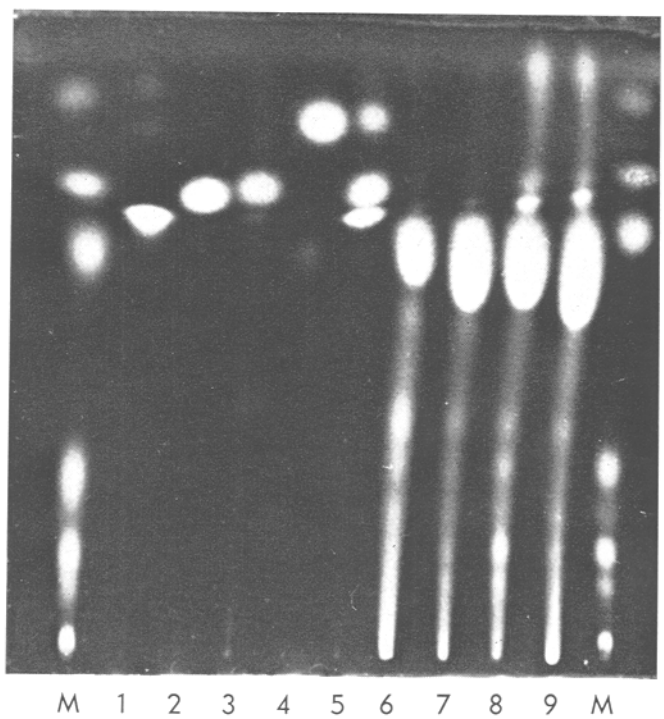

FIG. 3. TLC of known sterols and oxidized cholesterol samples. *M* is the sterol mixture of Fig. 1, (*1*) 1,4-cholestadien-3-one, (*2*) 4,6-cholestadien-3-one, (*3*) 5-cholesten-3-one, (*4*) cholestan-3-one, (*5*) 1–4 mixture, (*6*) NIH, (*7*) irradiated, (*8*) 175C heated (*9*) 8 weeks at 82C.

TABLE III
Summary of TLC Results

Name	Rf	Color[a]	Samples tested				
			Sample A	Sample B impurity	82C[b] heated	U.V. irradiated	175C heated
Cholestan-3β, 5α							
6β-triol	0.03	Brown	?	?	2X	?	?
7α-Hydroxycholesterol[c]	0.12	Blue	X	X	3X	2X	5X
7β-Hydroxycholesterol[c]	0.17	Blue	X	X	X	2X	3X
7-Ketocholesterol	0.23	Tan	2X	2X	3X	X	4X
Epoxycholesterol	0.31	Brown	2X	?	?	?	2X
Cholesterol	0.67	Purple	5X	>10X	>10X	>10X	>10X
1,4-Cholestadien-3-one	0.70	Light brown	X	?	3X	X	3X
4,6-Cholestadien-3-one	0.72	Tan					
5-Cholesten-3-one	0.73	Tan	X	X	2X	X	3X
4-Cholesten-3-one	0.75	Tan					
Cholesten-3-one	0.84	Tan	Nil	Nil	?	Nil	?
3,5-Cholestadien-7-one	0.87	Tan					

[a] After spraying with 50% H_2SO_4 and heating at 135C for 3–5 min.
[b] 8-week sample.
[c] Mixture obtained by reduction of 7-ketocholesterol.

5α, 6β-triol. Spot *b* is a mixture of α- and β-7 hydroxycholesterols with the β isomer having the higher R_f. Spot *c*, just barely visible, is 7-ketocholesterol. Spot *d* is epoxycholesterol, and spot *e* is cholesterol. Spot *f* is 4-cholesten-3-one, and near the solvent front is spot *g*, 3,5-cholestadien-7-one. Noteworthy in Fig. 1 is the decrease in size of the cholesterol spot as the heating progresses. Table III shows R_f values and the colors observed after heating the plate for 3–5 min. at 135C. If the plate is heated for 1 hr, the ketone spots become more plainly visible and all other spots become black in color. If the plate is merely sprayed with sulfuric acid and held at room temperature, only the blue spots from the hydroxycholesterol are visible. On this plate evidence for the formation of the hydroxycholesterols during heating at 82C is plainly visible. The amount of the α isomer appears to decrease as heating is continued. Also seen here is some indication of the possible presence of 7-ketocholesterol, epoxycholesterol, and 4-cholesten-3-one.

Fig. 2 shows the chromatogram obtained with the same series of seven knowns spotted as individuals (a-g) and as a mixture (M). Also included are the aged Sample A and the impurity from Sample B. Here is evidence for the presence of the diols, possibly 7-ketocholesterol, and the epoxide. There are at least four other unidentified spots in these aged samples.

Fig. 3 includes several additional known sterols. The M spots include the knowns (a-g) already described above. Spot 1 is 1,4-cholestadien-3-one, spot 2 is the 4,6-dien-3-one, spot 3 is 5-cholesten-3-one, spot 4 is cholestan-3-one, and spot 5 is a mixture of 1 through 4. Spot 6 is the aged cholesterol Sample A, spot 7 is a sample irradiated with ultraviolet light at room temperature for 3 days, spot 8 is a sample heated for 16 hr at 175C in air, and spot 9 is the 9-week sample at 82C. In these autoxidized mixtures is seen evidence for the presence of the diols, 7-ketocholesterol, cholesterol epoxide, the 1,4-dien-3-one, 4- or 5-cholesten-3-one, and the 4,6-dien-3-one. The evidence is much more convincing when the color of the various sterol spots is taken into account. TLC results are summarized in Table III.

Gas-Liquid Chromatography

As mentioned earlier, the sterols were first converted to their trimethylsilyl ethers before injection in order to avoid decomposition on the column which occurred particularly with the epoxide. Fig. 4 represents a composite of two chromatograms in which the ketones, samples 8–14 were chromatographed directly whereas 1–7 were injected as the TMS ethers. The pair 3,5-cholestadien-7-one with cholestan-3-one, and the pair 4-cholesten-3-one with 5-cholesten-3-one were not resolved.

Two of the 82C heated samples were then converted to their TMS ethers and chromatographed as shown in Fig. 5. In addition to the main cholesterol peak, these samples contain 7-β-hydroxycholesterol, the triol, epoxycholesterol, 3,5-cholestadien-7-one, probably 4,6-cholesetadien-3-one, 7-ketocholesterol, and 1,4-cholestadien-3-one. The other oxidized cholesterol samples in Table III were also silylated and assayed.

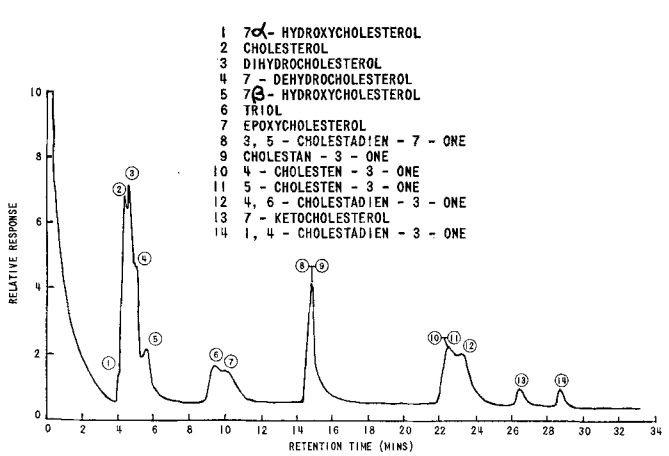

FIG. 4. Gas-liquid chromatography. Known steroids on QF-1 column at 220C.

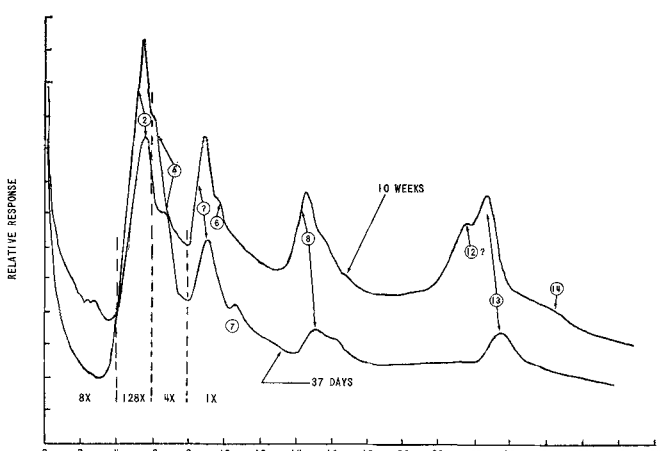

FIG. 5. Gas-liquid chromatography. Cholesterol heated at 82C.

TABLE IV

Summary of GLC Results[a]

Name	Relative retention time[b]	Samples tested					
		Sample A	Sample B impurity	82C Heated		U.V. irradiated	175C heated
				37 days	10 weeks		
Cholesterol[c]	1.00						
7β-Hydroxycholesterol	1.30	>1000X[d]	>100X	>1000X	>1000X	>1000X	>1000X
Cholestan-3β, 5α, 6β-triol	2.20	25X	100X	12X	10X	5X	>100X
Epoxycholesterol	2.36	>100X?	Nil	>100X?	>100X	50X	>100X
3,5-Cholestadien-7-one	3.32	?	?	4X	?	2X	12X
Cholestan-3-one	3.34	50X	30X	25X	100X	8X	70X
4-Cholesten-3-one 5-Cholesten-3-one	5.14	20X?	80X?	Nil	Nil	Nil	50X
4,6-Cholestadien-3-one	5.34	25X?	?	Nil	50X	Nil	20X
7-Ketocholesterol	6.00	>100X	>100X	75X	100X	>100X	>100X
1,4-Cholestadien-3-one	6.52	10X	10X	Nil	10X	Nil	Nil

[a] 6 ft-⅛ in. column of 3% QF-1 on 100/120 Gas-Chrom Q.
[b] At 220C and 60 ml He/min the time for cholesterol is 4.4 min.
[c] All hydroxy-containing compounds run as the TMS derivatives.
[d] X is the minimum detectable response at 10X attenuation; 50 μg total sample injected in each case.

The results of these GLC experiments appear in Table IV.

In Table V are summarized the results of this study showing both those autoxidation products of cholesterol identified by previous workers and the ones found here using a combination of TLC and GLC techniques. In addition to the known autoxidation products, the epoxide and 1,4-cholestadien-3-one were found in this study.

TABLE V

Oxidation Products of Cholesterol
Summary of Results

Name of compound examined	Previously identified	Found in this study
7α-Hydroxycholesterol[c]	X	X
Dihydrocholesterol[a,b]	X	X
7β-Hydroxycholesterol[c]	X	X
Cholestane-3β, 5α, 6β-triol[c]	X	X
Epoxycholesterol		X
3,5-Cholestadien-7-one Cholestan-3-one	X	X
4-Cholesten-3-one 5-Cholesten-3-one	X	X
4,6-Cholestadien-3-one	X	X
7-Ketocholesterol[c]	X	X
1,4-Cholestadien-3-one		X

[a] Not distinguishable from cholesterol.
[b] Originally present as an impurity.
[d] Major oxidation by-product.

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