THIN-LAYER CHROMATOGRAPHIC EXAMINATION OF CHOLESTEROL AUTOXIDATION

LELAND L. SMITH, W. STEPHEN MATTHEWS, JOHN C. PRICE, RICHARD C. BACHMANN AND BRIAN REYNOLDS
Department of Biochemistry, University of Texas Medical Branch, Galveston, Texas 77550 (U.S.A.)
(Received October 4th, 1966)

The great complexity of the air oxidation of cholesterol has been recognized for some years. Additionally it is known that cholesterol is unstable towards light, heat, and other radiation in the presence of air. As a consequence detection in or isolation from biological material of cholesterol autoxidation products has been suspect[1–4], and artifact status is frequently assigned these oxidized sterols. However, some have asserted that oxidized sterols, where found, are true components initially present in the tissue sample under investigation[5,6]. Reviews of cholesterol autoxidation have left the matter unsettled[7–9].

The full impact of the complexity of cholesterol autoxidation did not materialize so long as the usual isolation or colorimetric assay procedures were relied on. Paper chromatographic methods did suggest reaction product complexity[10] and tissue extract complexity[11–14], but the method was never exploited. With the advent of thin-layer chromatography detection of autoxidation products in sterol samples has been greatly simplified, and progress is being made in recognition and identification of the complex products of air oxidation[15–19].

The present paper details some of our recent experiences in the application of thin-layer chromatography to the study of cholesterol autoxidation.

EXPERIMENTAL

Materials

A number of U.S.P. and reagent grade cholesterol samples of several manufacturers and distributors (Eastman Kodak Co., Rochester, N.Y.; Fisher Scientific Co., Pittsburgh, Pa.; Merck and Co., Rahway, N. J.; Pfanstiehl Chemical Co., Waukegan, Ill.; Coleman and Bell Co., Norwood, Ohio; and others) variously received at the University between October 1948 and October 1964, were taken as unopened bottles from store room shelves at random. In addition a number of cholesterol samples isolated from bovine and human liver, adrenals, and aorta intimal tissue and stored in amber screw-cap glass bottles were examined. Cholesterol samples were examined "as is", and also after recrystallization from methanol, which afforded a concentrated autoxidation product fraction.

Solvents for chromatography

All solvents were of ordinary analytical reagent purity and were redistilled prior to use.

One-dimensional thin-layer chromatography

Chromatoplates 250 μm thick were prepared with Silica Gel H and HF_{254} (E. Merck AG, Darmstadt) with the Desaga Model S11 variable thickness spreading device on 5 × 20, 20 × 20, and 20 × 80 cm plates. The 5 × 20 cm plates were used for examination of one sample *versus* reference cholesterol, etc. and for photoelectric densitometric scanning. The 20 × 20 cm plates were used routinely for comparison of several samples. The 20 × 80 cm plates were used for extended irrigation times ascending (15 h irrigation time, run in the 80 cm dimension).

Sterol samples were applied at the start line 1 cm from one end as chloroform solutions using disposable capillary micropipettes (1, 2 and 5 μl volumes, Drummond Scientific Co., Broomall, Pa.). Irrigation in closed glass chambers lined with Whatman No. 1 filter paper was conducted at room temperature, ascending, with the solvent system ethyl acetate–heptane (1:1) for 30–40 min, at which time the solvent front had advanced to about 16 cm. Irrigated plates were dried momentarily in air and with a current of warm air prior to multiple irrigation with the same solvent (up to four separate irrigations) or to visualization.

Two-dimensional irrigation

Samples were spotted at a point 1 cm above the lower edge and 1 cm from the left edge of a 20 × 20 cm chromatoplate prepared with Silica Gel HF_{254} and irrigated in one dimension with ethyl acetate–heptane (1:1) in the usual manner. The chromatoplate was dried momentarily and reirrigated with the same solvent system, after which it was irrigated in the second dimension with acetone–heptane (1:1) twice. Further multiple irrigation, up to four irrigations in each system, improved resolution.

Visualization

Air dried chromatoplates were routinely examined under ultraviolet light (254 and 366 nm mercury lamps) prior to visualization. The standard visualization procedure consisted of spraying the chromatoplate, held at about 30° from the vertical, with (1) 50% aqueous sulfuric acid, followed by heating at 110–120° for 15 min, during which time the color display was noted.

Additional visualization procedures include: (2) 10% phosphomolybdic acid in 95% ethanol, heated 10 min at 105°; (3) 50% aqueous phosphoric acid, heated 5-10 min at 105°; (4) chlorosulfonic acid–acetic acid (1:2), heated 10 min at 105°; (5) 25% aqueous *p*-toluenesulfonic acid, heated 5 min at 105–110°; (6) 1% vanillin in 50% aqueous phosphoric acid, heated 10 min at 105°; (7) 25% aqueous perchloric acid, heated 10 min at 105°; (8) antimony trichloride, saturated solution in chloroform, heated 5–10 min at 105°; (9) the LIEBERMANN-BURCHARD reagent: acetic anhydride–sulfuric acid (4:1); (10) antimony pentachloride, 20% solution in carbon tetrachloride. Color display was noted in each instance.

Heating at 105–110° was necessary for full display of color with most of these reagents; however, heating beyond 15 min was not necessary for any test to bring out the full color development of all detected sterol components.

For qualitative detection of sterol components by charring, the plates were sprayed with 50% aqueous sulfuric acid and heated at 110° until an even brown-to-black zone developed against a white background. For quantitative evaluation of sterol components by charring a slightly modified acid reagent was used (see below).

Unsaturated ketosteroids were detected by means of their ultraviolet light absorption properties on Silica Gel HF_{254} plates under the 254 nm mercury lamp. Solutions of 2,4-dinitrophenylhydrazine (0.5% in 2 *N* hydrochloric acid) and of isonicotinic acid hydrazide (4 g per liter of methanol, to which 5 ml of concentrated hydrochloric acid was added) were also used for detection of unsaturated ketosteroids. Peroxide color tests were applied on separate chromatoplates (not sprayed with other reagents) using ammonium thiocyanate–iron(II) sulfate reagent and potassium iodide–starch reagent listed by WALDI (Nos. 5 and 85 respectively)[20].

Photoelectric densitometric estimation

Chromatoplates 5 × 20 cm and 250 μm thick were prepared in the usual manner with Silica Gel H. The assay sample dissolved in ethyl acetate (0.1–1.0 μg/μl concentration) was applied as a streak by multiple hand spotting using disposable 1 μl pipettes, so that a total sample of 1–10 μg in 10 μl was deposited as a zone 3 × 15 mm, which size covered the slit opening of the instrument. The chromatoplates were irrigated three times in sequence in one dimension using ethyl acetate–heptane (1:1) (solvent front rise: 16 cm). The chromatoplate was air dried and then dried at 110° in an oven to remove last traces of solvent residue. The dried plate was sprayed evenly with a freshly prepared 1:1 mixture of concentrated sulfuric acid and saturated aqueous ceric ammonium sulfate. The sprayed plate was heated at 110° on a hot plate for 10 min to produce an uniform brown-black char. The plate was cooled to the touch and scanned immediately with a Photovolt Corporation photoelectric densitometer Model 501, the scanning head of which was equipped with a violet filter (465 nm). Scanning was done manually and meter readings taken every 1 mm were recorded on chart paper point by point, the zero being set on the densitometer using a portion of the treated chromatoplate which had been irrigated by the solvent system but which was devoid of organic (charred) components. A blank scan of treated chromatoplates (without sampled applied) uniformly produced a flat, featureless curve.

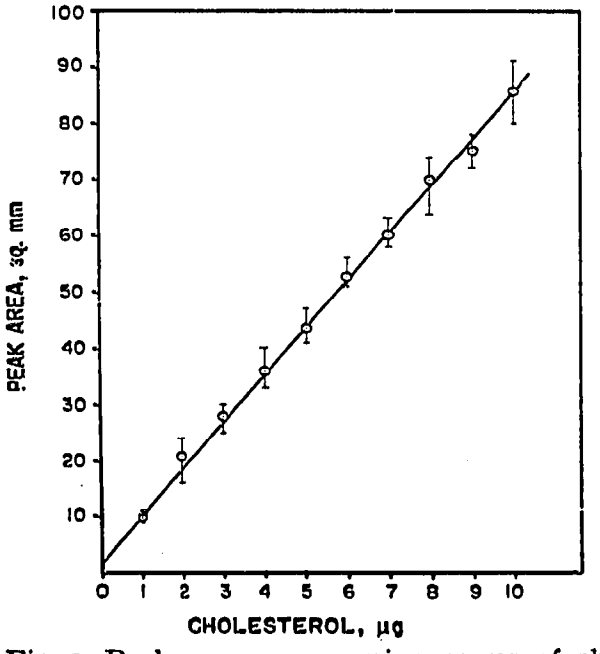

Fig. 1. Peak area *versus* micrograms of cholesterol, as assayed by photoelectric densitometer.

The area under resolved peaks obtained by the point-by-point plot of densitometric data *versus* distance moved on the chromatoplate was determined by counting squares or by triangulation. A plot of peak area in square millimeters *versus* micrograms of cholesterol gave a linear relationship (Fig. 1) over the range (0–10 μg) examined. By converting abscissa units to micromoles of C_{27}-sterol (or micromoles of carbon) it was possible to demonstrate that other reference C_{27}-sterols (7-ketocholesterol, 25-hydroxycholesterol, 5α-cholestane-3β,5,6β-triol, cholesta-3,5-dien-7-one, and 5α-cholestan-3β-ol) gave responses exactly equivalent to that of cholesterol.

One assistant working a nominal eight hour day can readily run twenty such assay samples operating the complete procedure of sample preparation, spotting, irrigation, charring, and densitometric evaluation. The major time consuming step of the operation was the manual photoelectric densitometric scanning.

Special samples

Several special samples of cholesterol were prepared by different purification and oxidizing reaction conditions.

A cholesterol sample of very high purity was prepared by the bromination–debromination procedure of FIESER[21]. A 38.7 g batch of U.S.P. cholesterol yielded 28.84 g of once purified cholesterol, m.p. 148–150° (all melting points taken on a Kofler block under microscopic magnification), chromatographically homogeneous. A 19.4 g portion of this sample was processed a second time by FIESER's procedure and recrystallized from methanol, yielding 14.25 g of purified cholesterol, m.p. 149.0–149.5°. One year later the sample had developed an acrid odor and was again twice brominated and debrominated and twice recrystallized from methanol, affording colorless, odorless crystals, m.p. 149.0–150.0°, homogeneous on paper chromatography using the 2-phenoxyethanol–heptane system of NEHER AND WETTSTEIN[22]. The sample was stored in an amber screw-cap glass bottle at ambient cellar temperatures for twelve years, after which time the sample was characterized as off-white colored needles, m.p. 138.0–140.0° (with sweating from 125°), bearing an acrid odor and shown to contain numerous oxidation products by thin-layer chromatography.

"Oxycholesterol", an undefined sterol oxidation product of LIFSCHÜTZ, was prepared using his early directions[7,23]. Benzoyl peroxide (2 g) was added to a heated (90°) solution of 20 g of pure cholesterol in 100 ml of glacial acetic acid, after which the mixture was brought to boiling. The mixture was poured into 200 ml of distilled water, filtered, and the products saponified with 0.5 *N* alcoholic potassium hydroxide solution. Sterols were extracted with ether, the ether extracts washed, dried, and evaporated under vacuum, and the sterol residue was examined chromatographically. As a control a similar solution of cholesterol in acetic acid was treated with benzoyl peroxide but no heat was employed at any time.

Purification of cholesterol samples *via* the dibromide was accomplished by the procedure of FIESER[21]. The purified cholesterol was recrystallized from methanol, and the methanol mother liquor was examined for sterol oxidation products.

Colloidally dispersed cholesterol preparations according to the conditions of BERGSTRÖM AND WINTERSTEINER[24,25] were obtained by adding 250 mg of cholesterol (purified *via* the dibromide and recrystallized from methanol) in 50 ml of 95% ethanol to a dispersal medium composed of 32.2 mg of sodium stearate in 100 ml of distilled water (all at room temperature). Buffering was accomplished by adding

3.0 ml of 0.4 *M* disodium hydrogen phosphate and titrating with 0.4 *M* sodium dihydrogen phosphate to the selected pH within the range pH 7.0–8.4. For pH within the range 4.8–6.0, 3.0 ml of 1.0 *M* sodium acetate was titrated with 2 *N* acetic acid; for pH 4.8, 3.0 ml of 0.4 *M* disodium hydrogen phosphate titrated with 0.1 *M* phosphoric acid; for pH 2.0, 3.0 ml of saturated calcium oxalate solution titrated with 1.0 *M* oxalic acid.

Metal catalysis was obtained by adding 5 mg of either copper(II) chloride dihydrate or iron(II) sulfate to each reaction mixture. Blank preparations without sodium stearate were made in all cases.

Experimental dispersal medium using 32.2 mg of crystallized bovine serum albumin (Pentex Incorporated, Kankakee, Ill.) in place of sodium stearate was examined. Additional dispersions were made by substituting for sodium stearate 328.7 mg of human whole plasma (buffered with 1.0 ml of 0.1 *M* sodium carbonate +. 2.0 ml of 0.4 *M* sodium dihydrogen phosphate, adjusted to pH 7.4) and 32.2 mg of lyophyllized human plasma (similarly buffered to pH 7.4).

The dispersed preparations were aerated with compressed air and agitated in a water bath at 37°, 50°, and 85° for suitable time periods. Samples (5 ml) were withdrawn at intervals, acidified, extracted with 1 ml of methylene chloride, and 50 μl of methylene chloride extract was chromatographed on one-dimensional chromatoplates using ethyl acetate–heptane (1:1). Those samples showing autoxidation were then run in two-dimensional chromatography using ethyl acetate–heptane (1:1) and acetone–heptane (1:1).

Cholesterol suspensions for several autoxidation experiments were prepared by the method of DAY *et al.*[26] wherein 500 mg of purified cholesterol in warm acetone was fed a bit at a time (under vacuum) to 200 ml of water in a rotary vacuum evaporator. After complete removal of the acetone under vacuum (no heat) the aqueous cholesterol dispersion was examined by thin-layer chromatography and utilized as such for other experiments.

Heat promoted air oxidation of cholesterol was obtained by storing clear glass vials of cholesterol, purified via the dibromide and recrystallized several times from methanol, m.p. 149.5–150.5° in contact with air (glass wool plugged) at different temperatures. Samples were held at 0–5°, at ambient room temperature (*ca.* 22°), and at 65° (in an electric oven). A control sample in an amber screw-cap bottle was maintained in the freezer. Assay samples from each were taken at weekly intervals for thin-layer chromatographic analysis.

Ultraviolet light promoted air oxidation of cholesterol was obtained by depositing 140 mg of the purified cholesterol sample as a methanol solution onto a 5 × 20 cm glass plate. After evaporation of the methanol the cholesterol film was placed 10 cm from a germicidal mercury lamp (254 nm) (General Electric G8T5 tube) and irradiated for 5 h at which time the irradiated sterol was examined chromatographically. Control films were exposed to ordinary daylight within the lab. for 5 h and to fluorescent white lamps (10 cm distance) for 5 h.

RESULTS

Initial thin-layer chromatographic examination of cholesterol samples from various sources indicated that those companion sterols and autoxidation products

TABLE I

ONE-DIMENSIONAL RESOLUTION OF AUTOXIDATION PRODUCTS IN NATURALLY AIR-AGED SAMPLES

Spot No.	R_c[a]	*Color display with acidic reagents*[b]								*Peroxide test*[b]	*Tentative identity*
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*		
1	1.1	be	bl	—	—	—	—	—	—	(—)	cholesta-3,5-dien-7-one
2	1.00	mag	bl	mag	mag	mag	r	r	mag	(—)	cholesterol
3	0.88	w	bl	w	—	—	—	—	—	(—)	
4	0.81	v	bl	w	r	r	w	br	—	(+)	
5	0.73	w	bl	w	—	—	—	—	—	(—)	
6	0.66	v-ol	bl	w	bl-r	r	r	br	w	(+)	25-hydroxycholesterol
7	0.63	—	—	—	—	—	—	—	—	(—)	7-ketocholesterol[c]
8	0.54	bl-v	bl	—	—	y	—	—	—	(—)	
9	0.43	bl	bl	bl	bl	bl	bl	bl	bl	(+)	7β-hydroxycholesterol
10	0.36	bl	bl	bl	bl	bl	bl	bl	bl	(+)	7α-hydroxycholesterol
11	0.25	y	bl	w	w	w	y	—	—	(+)[d]	
12	0.17	br[d]	bl[d]	—	br[d]	br-r[d]	—	—	—	(+)[d]	
13	0.11	br[d]	bl[d]	—	br[d]	br-r[d]	—	—	—	(+)[d]	
14	0.08	br[d]	bl[d]	w	br[d]	br-r[d]	br	bl	—	(+)[d]	5α-cholestane-3β,5,6β-triol
15	0.00	br	bl	w	br	br-r	br	—	—	(+)	

[a] Mobility relative to cholesterol as unity in the ethyl acetate–heptane (1:1) system.

[b] Reagents as defined in the Experimental section. Colors abbreviated: be = beige; bl = blue; mag = magenta; r = red; w = white, fluorescence; v = violet; ol = olive; y = yellow, fluorescence; br = brown.

[c] Detection by ultraviolet light absorption only.

[d] Streaked back to origin.

which were detected with sulfuric acid and with phosphomolybdic acid were generally more polar than cholesterol. Accordingly solvent systems were devised which placed cholesterol at a relatively high R_F but which would still resolve the more mobile autoxidation product cholesta-3,5-dien-7-one from cholesterol. The system ethyl acetate–heptane (1:1), patterned after the ethyl acetate–hexane (1:1) system previously used for cholesterol on starch-bound silica gel chromatoplates[27], gave excellent resolution of many of the known oxidation products of cholesterol. The related acetone–heptane (1:1) system gave improved resolution of the more polar cholesterol oxidation products but still resolved cholesta-3,5-dien-7-one from cholesterol at relatively high R_F values.

Application of either solvent system in ascending uni-dimensional development to complex cholesterol autoxidation mixtures regularly afforded resolution of about fifteen sterols, including evidence of the familiar oxidation products: cholesta-3,5-dien-7-one, 25-hydroxycholesterol, 7-ketocholesterol, 7β-hydroxycholesterol, 7α-hydroxycholesterol, 3β,5-dihydroxy-5α-cholestan-6-one, and 5α-cholestane-3β,5,6β-triol (in order of decreasing mobility).

Complete resolution of these seven sterols was possible in either system with the usual uni-dimensional irrigation (using reference sterols). However, in autoxidation mixtures from almost any source it was apparent that unresolved sterols were present and that one-dimensional irrigation in these systems was inadequate to the task of complete analysis. The region occupied by 25-hydroxycholesterol and 7-ketocholesterol was particularly complex, as witnessed by variable resolution of components and variable color test response with different samples. From reference sterol mobility data it was obvious that sterols potentially implicated in cholesterol autoxidation migrated into this region.

25-Hydroxycholesterol is slightly more mobile than 7-ketocholesterol and can be resolved from it by one-dimensional irrigation, with improved resolution obtaining on multiple irrigation. Additionally, 25-hydroxycholesterol is readily differentiated from 7-ketocholesterol by its color response (an initial magenta color which changes rapidly to red-violet, finally to a grey-blue color which persists) to 50 % sulfuric acid. 7-Ketocholesterol does not give a color response and can be detected by this means only by charring, but it is easily detected (to as little as 0.025–0.05 μg) by ultraviolet light absorption properties, while 25-hydroxycholesterol is not detected with any sensitivity by ultraviolet light absorption techniques.

25-Hydroxycholesterol and cholest-5-ene-3β,4β-diol are not resolved well however, nor are 25-hydroxycholesterol and 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide well resolved. Both the 3β,4β-diol and the 5α-hydroperoxide have been implicated in prior studies of cholesterol autoxidation, although their confident recognition in the present studies has not been achieved.

One-dimensional ascending chromatography for extended times on an 80 cm chromatoplate, including multiple irrigation, did not offer the resolution sought. Accordingly, in order to improve resolution of additional sterol components in the more complex mixtures, a two-dimensional irrigation of the chromatogram using the ethyl acetate–heptane (1:1) and acetone–heptane (1:1) systems was explored. Vastly improved resolution of sterol components resulted, and in complex samples yielding only fifteen components in one-dimensional irrigation (Table I) there were resolved over thirty sterol components by the two-dimensional technique. Furthermore the

two-dimensional procedure gave clear evidence of the additional (unresolved) complexity of the most polar regions of the chromatogram. The two-dimensional technique nevertheless still did not offer complete resolution of 25-hydroxycholesterol from 3β-hydroxy-5α-cholest-6-en-5-hydroperoxide.

Results obtained on two-dimensional chromatography of a twelve-year old cholesterol sample are presented in Table II and in Fig. 2. Again the presence of the seven known autoxidation products cholesta-3,5-dien-7-one, 25-hydroxycholesterol, 7-ketocholesterol, 7β-hydroxycholesterol, 7α-hydroxycholesterol, 3β,5-dihydroxy-5α-cholestan-6-one, and 5α-cholestane-3β,5,6β-triol was clearly demonstrated

TABLE II

AUTOXIDATION PRODUCTS OF CHOLESTEROL RESOLVED BY THIN-LAYER CHROMATOGRAPHY

Spot No.	*Visible color*[a]	*Relative intensity*[b]	R_F		*Tentative identity*
			(I)[c]	*(II)*[d]	
1		intense	1.00	1.00	solvent front materials
2	violet	weak	0.90	0.83	
3	beige	weak	0.86	0.77	cholesta-3,5-dien-7-one
4	violet	faint	0.80	0.83	
5	magenta	intense	0.78	0.73	cholesterol
6	violet	weak	0.70	0.65	
7	violet	medium	0.68	0.61	
8	blue[e]	medium	0.64	0.69	
9	violet	weak	0.58	0.55	
10	magenta to grey-green	strong	0.54	0.60	25-hydroxycholesterol
11	-[f]	strong	0.49	0.62	7-ketocholesterol
12	grey-violet	weak	0.49	0.53	
13	grey	weak	0.43	0.59	
14	violet	weak	0.43	0.50	
15	blue[e]	medium	0.38	0.54	7β-hydroxycholesterol
16	blue[e]	medium	0.35	0.54	7α-hydroxycholesterol
17	violet	medium	0.29	0.48	
18	yellow	medium	0.28	0.54	3β,5-dihydroxy-5α-cholestan-6-one
19	yellow	medium	0.23	0.45	
20	violet	medium	0.15	0.39	
21	yellow	strong	0.08	0.32	5α-cholestane-3α,5,6β-triol
22	violet	weak	0.17	0.26	
23	beige	weak	0.06	0.25	
24	beige	medium	0.03	0.20	
25	brown	medium	0.03	0.16	
26	violet	medium	0.12	0.14	
27	brown	medium	0.01	0.10	
28	violet	medium	0.08	0.08	
29	brown	intense	0.01	0.02	
30	brown	strong	0.04	0.04	
31	brown	intense	0.04	0.01	
32	brown	intense	0.00	0.00	origin components

[a] With 50% aqueous sulfuric acid

[b] Relative intensity of the spot after complete charring following spraying with 50% sulfuric acid.

[c] First dimension irrigated with ethyl acetate–heptane (1:1).

[d] Second dimension irrigated with acetone–heptane (1:1).

[e] Blue color develops immediately after spraying with sulfuric acid prior to heating.

[f] Detected by means of ultraviolet light absorption properties. A questionable grey or violet color developed in some samples.

in the complex sample. Multiple irrigation, up to four separate irrigations in each dimension, sharpened resolution over the central regions of the chromatoplate.

In no instance was there evidence that the additional sterols resolved in two-dimensional irrigation were artifacts of the chromatography.

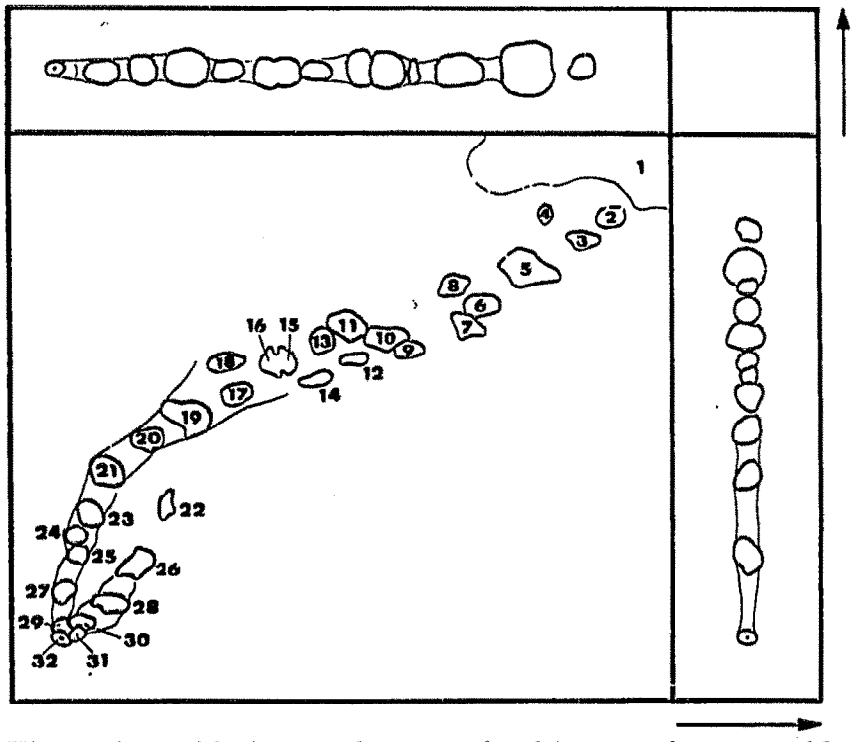

Fig. 2. Autoxidation products resolved in a twelve-year old, specially purified cholesterol sample. In this and other chromatogram charts the initial irrigation with ethyl acetate–heptane (1:1) is the horizontal dimension, irrigation with acetone–heptane (1:1) is the vertical dimension. For explanation of numbers, see Table II.

The exact location of the major autoxidation products 7-ketocholesterol and the epimeric 7-hydroxycholesterols on paper and thin-layer chromatograms has been subject to confusion in some prior reports. Thus, HORVATH located 7-ketocholesterol ahead of cholesterol in his recent studies of cholesterol stability using the thin-layer chromatographic system chloroform–acetone (5:2)[19]. Our examination of this system on Silica Gel HF_{254} placed 7-ketocholesterol at R_F 0.40, thus more polar than cholesterol (at R_F 0.53), which result is more in line with usual experience.

The relative mobilities of the epimeric 7-hydroxycholesterols on partition chromatographic systems were shown early to conform to the generalization that the axial epimer is the more mobile[28]. Thus, SMITH[10] using the 2-phenoxyethanol–heptane paper chromatographic system[22] (later confirmed by SCHUBERT *et al.*[29]) and MOSBACH *et al.*,[30] using propylene glycol–cyclohexane partition columns established that the 7α-epimer (pseudo-axial) was the more mobile of the 7-hydroxycholesterols.

The interim report of RIDDELL AND COOK[14], also using the 2-phenoxyethanol–heptane paper chromatographic system, placed 7β-hydroxycholesterol ahead of 7α-hydroxycholesterol. However, when proper account is taken of their use of the "old" nomenclature for these sterols (which reverses the configurational assign-

ments as used by RIDDELL AND COOK) their mobility data[31] also conforms with that of others. Unfortunately data of RIDDELL AND COOK[14] and of MOSBACH *et al.*[30] have been included in several important reviews and monographs without revision of nomenclature[32,33].

A reverse order of chromatographic mobility obtains for the 7-hydroxycholesterol epimers using adsorption procedures. The early column separation of the epimers (again requiring reversal of configuration for proper comparison[34]) and recent thin-layer chromatography on silica gel[17,18,35] clearly place 7β-hydroxycholesterol ahead of its 7α-epimer. Also at least one instance has been reported where the equatorial 7β-hydroxy epimer of 7-hydroxyprogesterone precedes the axial 7α-hydroxyprogesterone in a paper partition system[36]. Obviously care must be exercised in applying chromatographic mobility, whether in partition or in adsorption systems, to the assignment of configuration in 7-hydroxysteroids.

Although the present studies were not planned as quantitative, it was of value to have at hand a rapid semi-quantitative method for estimation of sterol composition in select preparations. Quantitation by spot intensity matching could be applied to the two-dimensional chromatograms, as could elution and spectrophotometric estimation.

However, the photoelectric densitometric evaluation of spot intensity after charring with sulfuric acid offered to us the most attractive proposition, even though two-dimensional irrigation could not be utilized in the procedure. Accordingly, we devised a modification of the densitometric methods of PEIFER[37] and PRIVETT *et al.*[38,39] for application to one-dimensional thin-layer chromatography using ethyl acetate–heptane (1:1).

In order to estimate levels of different sterols and to avoid unnecessarily demanding procedures no attempt was made to assay sterols on the basis of colors developed with sulfuric acid, rather densitometric evaluation of the uniform char produced with sulfuric acid containing ceric ammonium sulfate was made. Under the specified conditions equivalent densitometric responses were obtained with the several C_{27}-sterol derivatives over the concentration range 0–0.025 μmoles (0–10 μg of cholesterol).

Whereas prior methods of this sort utilized a single sample application as a spot, which moved in chromatographic irrigation as a rounded spot, we explored the use of a sample applied as a rectangular zone of the approximate size of the slit in the photoelectric densitometer. The sterol sample migrated as a band under these conditions. It has been emphasized that a second slit (one on each side of the chromatoplate) must be used to reduce the effects of scattered or refracted light[39]. Using our band migration technique and a single slit on the light source side of the instrument we were able to obtain satisfactory analytical results without modifying the densitometer search unit. However, the calibration curve did not pass directly through the origin but crossed the ordinate at a value of about 2 sq. mm, equivalent to about 0.25 μg of cholesterol (Fig. 1), thus indicating the extent of the error possible due to light scattering and difraction. These results were obtained using a violet light filter, which improved instrumental sensitivity.

The present simplified procedure is time-consuming and of limited precision, but could be improved by use of more sophisticated automated equipment. However, the procedure as described permits useful analyses to be made on complex sterol mixtures using relatively primitive apparatus.

DISCUSSION

The well recognized instability of cholesterol towards oxidative attack to give a "galaxy of more polar products"[4] is amply demonstrated by the present results. By utilizing thin-layer chromatographic means in place of isolation procedures or of the generally less sensitive paper chromatographic techniques it is possible to show the rapid formation of oxidation products in pure cholesterol held in air at room temperature for but a short time period. Additionally the very great complexity of several autoxidation reactions of cholesterol which occur under relatively mild conditions has been established by these chromatographic studies.

Our present examination of a series of reactions of cholesterol known from prior work to give oxidation products (either as specific sterol autoxidation products or as the poorly defined "oxycholesterol" of LIFSCHÜTZ) was initiated to explore the extent of chemical treatment possible before known oxidation reactions occurred. These controlled chemical reactions together with direct air oxidation of aged, heated, or irradiated cholesterol samples point out decisively the importance of exacting control analyses on all biochemical work with cholesterol, such control work being readily accomplished with the thin-layer chromatographic techniques herein described.

The oxidation reaction conditions examined include: (1) oxidation by benzoyl peroxide in acetic acid, the classic method for "oxycholesterol" of LIFSCHÜTZ[7,23]; (2) oxidation during purification *via* the dibromide; (3) autoxidation of colloidally dispersed cholesterol in sodium stearate media; (4) autoxidation by heating in air; (5) autoxidation of cholesterol films on irradiation by a 254 nm mercury lamp; (6) natural aging of cholesterol in contact with air.

In each of these instances examined a complex, discrete pattern of oxidation products was obtained, with no one pattern exactly duplicating another although some common features were present. The presence of the epimeric 7-hydroxycholesterols was demonstrated in each experiment. Thus of the several autoxidation products of cholesterol the 7-hydroxycholesterols appear to be the most likely encountered. The other important products 7-ketocholesterol and 5α-cholestane-3β,5,6β-triol were not detected in every case, and 25-hydroxycholesterol was detected only in those cases involving solid cholesterol samples.

In this respect the uniqueness of 25-hydroxycholesterol as a product of air oxidation of solid cholesterol has been commented on by BECKWITH[40], who suggested that the course of the reaction was governed by the arrangement of the sterol molecules in the crystal. While we did not detect 25-hydroxycholesterol in air oxidation of colloidally dispersed cholesterol, BECKWITH did encounter 25-hydroxycholesterol in the oxidation by Fenton's reagent of water suspensions of cholesterol[40].

By means of relative mobility *versus* reference sterols, ultraviolet light absorption properties on chromatoplates, and color responses to 50 % sulfuric acid it was possible to classify the several resolved autoxidation sterols into tentative structural categories: (1) Δ^5-3β-ols, characterized by their red, magenta, red-violet, violet, grey-violet, or grey-green colors with sulfuric acid; (2) Δ^5-7-ols, characterized by their immediate intense sky blue colors with sulfuric acid; (3) unsaturated 7-ketones, characterized by their weak or absent color response to sulfuric acid and by their strong ultraviolet light absorption properties; (4) doubly oxidized sterols, including

those oxidized at the C-5,6 double bond, triols, etc., characterized by their low chromatographic mobilities and by their yellow or brown color response to sulfuric acid. These four categories account only for those sterols actually resolved. The several known autoxidation products of cholesterol all fall into their appropriate category. In addition there are sterol peroxides as detected with the standard iodide–starch and iron(II) thiocyanate reagents; however, these peroxides have not been adequately differentiated by their color response to sulfuric acid and are, therefore, not included as a separate category. Furthermore, in ordinary cholesterol samples not otherwise purified to insure their removal there may also be present the companion sterols 5α-cholestan-3β-ol, 5α-cholest-7-en-3β-ol, cholesta-5,7-dien-3β-ol, etc. which may or may not contribute to additional oxidation components.

The several findings of ready autoxidation of cholesterol will be considered individually:

(1) *Benzoyl peroxide oxidation*

Preparation of LIFSCHÜTZ's "oxycholesterol" mixtures by means of this reagent was considered to be one of the most direct means of linking present work with the many reports by LIFSCHÜTZ[23]. This reaction has long been recognized as being very complex and has been avoided accordingly. At least eight sterol components were resolved by our two-dimensional procedures (Fig. 3). 25-Hydroxy-

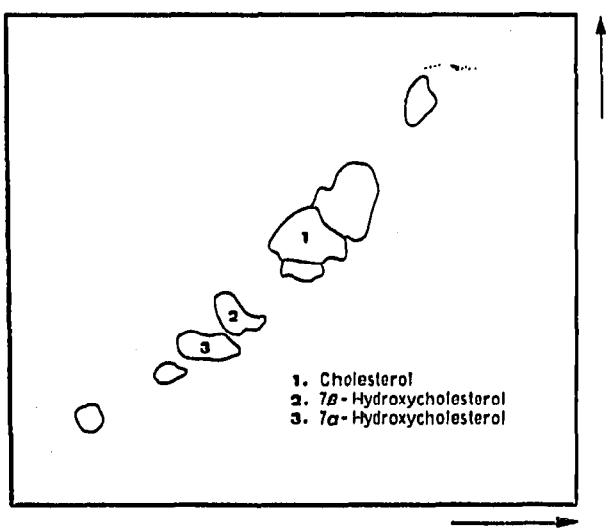

Fig. 3. Oxidation products detected in "oxycholesterol" prepared according to LIFSCHÜTZ[7,23].

cholesterol and 7-ketocholesterol were notable by their absence. However, the epimeric 7-hydroxycholesterols and several other sterols giving an immediate intense sky blue coloration with sulfuric acid were present. Reaction at room temperature without reflux also resulted in a very complex mixture of oxidized sterols not further studied.

These results amply justify the caution expressed by others in attempting to evaluate LIFSCHÜTZ's early work. With such variability between benzoyl peroxide attack on cholesterol depending on temperature, little hope can be expressed for linking any of LIFSCHÜTZ's work with this reaction to his or other work with biological systems yielding "oxycholesterol".

(2) *Oxidation via the dibromide*

Purification of cholesterol via its 5,6-dibromide is an important method of great utility in separating cholesterol from many of its companion sterols. However, the reaction is complex, the initially formed 5α,6β-dibromide is subject to mutarotation[41], and "oxycholesterol" formation[42] and autoxidation[10] is known to occur during work-up. Our present thin-layer chromatographic examination of the reaction established that at least eight sterol products were formed on debromination with zinc dust of the sterol dibromide (the dibromide itself contained six resolved sterol components). Chief among these products were the epimeric 7-hydroxycholesterols (Fig. 4). Identification of the other components by comparison of mobilities and color test responses *versus* reference samples was not possible. In this regard, these results confirm previous work with the bromination–debromination reaction using paper chromatography[10].

(3) *Colloidally dispersed systems*

We had occasion to repeat several of the classic experimental methods of BERGSTRÖM AND WINTERSTEINER using sodium stearate colloidally dispersed systems for the air oxidation of cholesterol[7,24,25]. In our thin-layer chromatographic examination of these reactions we found that either extensive autoxidation occurred, as evidenced by the formation of 10–15 sterol oxidation products, or else essentially no autoxidation occurred. None of the several conditions examined afforded reaction product mixtures of simple composition; rather, the complex spate of products was observed if any reaction occurred.

Autoxidation of colloidally dispersed cholesterol did not occur up to 170 h at 37° even with copper(II) ion catalysis. However, extensive autoxidation occurred at 50° (pH 7.4–8.4) with copper(II) ion catalysis. Iron(II) ion catalysis was much less effective in catalyzing the reactions at 50°. Additionally, at low pH (between pH 2.0–7.0) at 50° there were no detectable autoxidation products produced even though

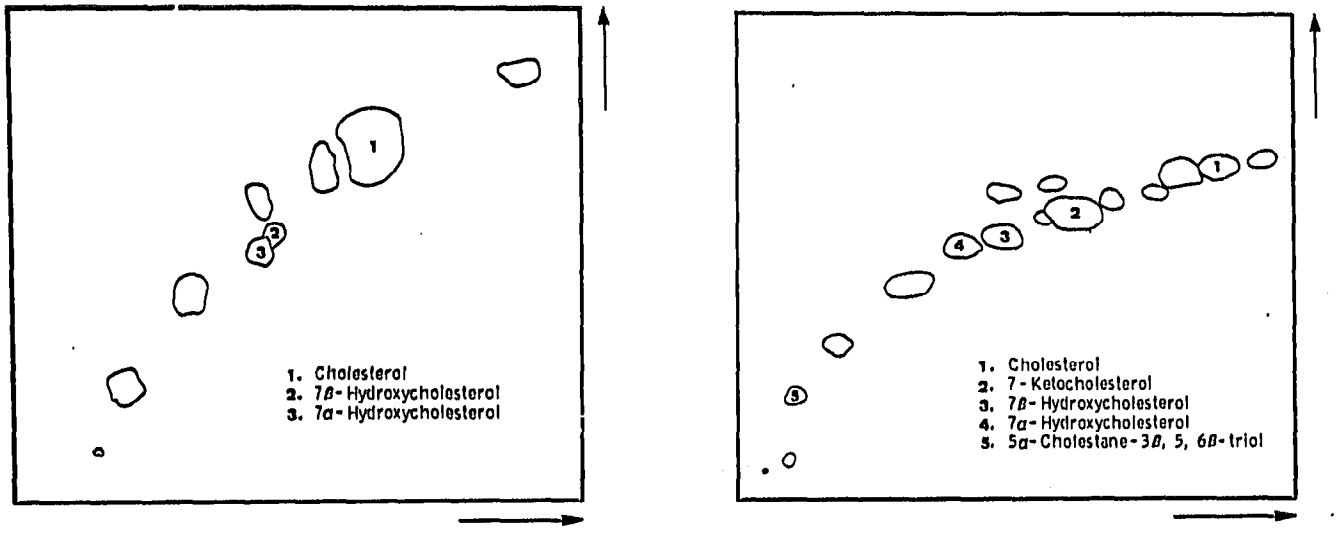

Fig. 4. Oxidation products detected in cholesterol purified via the dibromide.

Fig. 5. Cholesterol autoxidation products formed in sodium stearate dispersion, pH 8.2, at 85°, copper catalyzed.

copper(II) ion was present. These results confirm essentially the results of BERGSTRÖM AND WINTERSTEINER.

At elevated temperature (85°) extensive autoxidation occurred without addition of metal salts, yielding on two-dimensional chromatoplates a pattern (Fig. 5) of fifteen sterol autoxidation products, four of which (the epimeric 7-hydroxycholesterols, 7-ketocholesterol, and 5α-cholestane-3β,5,6β-triol) were known to form in these systems from the prior work of BERGSTRÖM AND WINTERSTEINER[7,24,25] and of MOSBACH *et al.*[30].

In terms of the induction period associated with cholesterol autoxidation in sodium stearate dispersions, we found an induction period of about one hour in experiments with pure cholesterol at 85° (see Fig. 6) whether catalyzed or not. The extent of autoxidation in the copper(II) ion catalyzed reaction at 85° proceeded to about 82–84 % oxidized, with 16–18 % of the reactant cholesterol unoxidized.

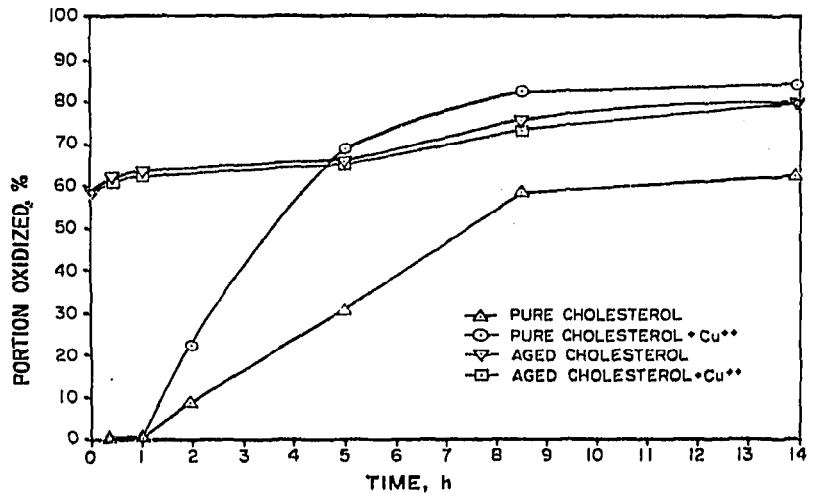

Fig. 6. Demonstration of an induction period in the autoxidation of cholesterol in sodium stearate colloidal dispersion.

In uncatalyzed reaction the extent of oxidation was less, stabilizing at between 59–63 % oxidized. In comparison experiments using an aged Eastman sample of cholesterol, assayed to contain about 60 % oxidation products, there was no clearly defined induction period, the reaction proceeding directly but slowly, with an increase of oxidation to about 78 %. These experiments confirm the induction period for the reaction specified by BERGSTRÖM AND WINTERSTEINER, the apparent product inhibition of the reaction, and additionally demonstrates the autocatalytic nature in the autoxidation products themselves on the oxidation reactions of cholesterol in colloidally dispersed media.

In none of these experiments was there found a trace of a sterol component which occupied the position of 25-hydroxycholesterol on the chromatoplates. We conclude that 25-hydroxycholesterol is not formed in the colloidally dispersed medium.

In examining additional possibilities for autoxidation of cholesterol in systems potentially encountered in biochemical work we attempted to observe the autoxidation of cholesterol in dispersions formed by means of protein rather than sodium stearate. Thus bovine serum albumin dispersions of cholesterol, at pH values between 2.0–8.4 and including pH 4.8 near the isoelectric point of the albumin, were treated

under conditions known to give extensive autoxidation of cholesterol in sodium stearate dispersions, both with and without copper(II) and iron(II) ion catalysis, at 27°, 50° and 85°. In no instance was there any detectable oxidation product formed. Autoxidation also did not occur when human whole plasma or lyophyllized human plasma was substituted for sodium stearate as dispersing agent. It is noteworthy that earlier workers similarly found that protein-dispersed cholesterol was not oxidized[43], although cholesterol and "oxycholesterol" may be associated with select plasma protein fractions[44]. Indeed, some proteins inhibit air oxidation of cholesterol in sodium stearate dispersion[7].

Of particular concern was the possibility of oxidation of cholesterol dispersed in aqueous phase by the addition of an organic solvent solution of cholesterol to water, with subsequent removal of the organic solvent, a condition recently utilized by DAY *et al.*[26] in preparing stable cholesterol suspensions for other studies. In our hands such cholesterol dispersions prepared from acetone or from ethanol were stable and did not contain demonstrable amounts of autoxidation products. Such solvent–water dispersions of cholesterol heated in air do lead to oxidation however.

(*4*) *Autoxidation by heating in air*

By merely heating pure cholesterol in air it is possible to decompose the sterol to an odorous, colored mass. Thin-layer chromatographic analysis of cholesterol samples subjected to heat for varying lengths of time affords an oxidation product distribution pattern which includes the epimeric 7-hydroxycholesterols and 25-hydroxycholesterol. Thus, heating pure cholesterol at 65° for one month led to the formation of an odorous, yellow colored sample which contained (besides cholesterol at R_C 1.00) components at R_C 0.8, 0.6 (25-hydroxycholesterol), 0.4 and 0.3 (the epimeric 7-hydroxycholesterols), and 0.08 (5α-cholestane-3β,5,6β-triol), with streaking from R_C 0.1 back to the origin (unidimensional analysis). Control samples at room temperature developed in the same time trace amounts of 25-hydroxycholesterol, while control samples at 4° did not develop any detectable oxidation products.

Recently HORVATH has published other studies of the stability of cholesterol to heat, wherein he found some 50% destruction of cholesterol after one week at 98° in air[19]. The very complex decomposition of cholesterol by heating in air has been known for many years[43,45,46]. However, ours and HORVATH's studies are the first attempts at examination of the remarkably complex oxidative reaction using modern methods.

(*5*) *Autoxidation on irradiation*

The action of light and other radiation on cholesterol in air has been known for a long time as a means of causing decomposition. More recently the role of exposure to light in the decomposition of cholesterol in biological experiments has been emphasized[47], and both the 7-hydroxycholesterols and 25-hydroxycholesterol have been implicated in photooxidation of cholesterol in other work. In our examination of cholesterol spread as a film on glass plates and irradiated by a 254 nm mercury lamp for short times we found these three sterols present, together with more polar components giving a brown color test with sulfuric acid and suspected of being oxidized at the C-5,6 double bond. (Fig. 7).

(6) Natural air aging of cholesterol

By far the most interesting and complex example of cholesterol autoxidation which we have encountered is that of natural air aging of cholesterol. Our one-dimensional chromatographic analysis of naturally air-aged cholesterol samples established the very complex nature of the processes, with over fifteen sterols being resolved. The one-dimensional chromatogram of these aged sterols is very similar in some respects to work of HORVATH using another solvent system and heat-decomposed cholesterol[19].

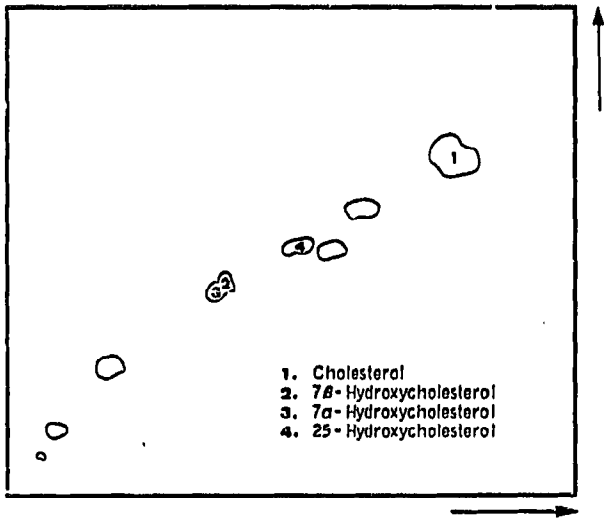

Fig. 7. Ultraviolet-light induced oxidation of cholesterol.

We have confirmed the presence of sterol peroxides in air-aged samples of cholesterol (Table I) as implicated earlier by NEUWALD AND FETTING[16]. The positive peroxide responses indicated in Table I for Component No. 6, identified as 25-hydroxycholesterol, clearly are not given by 25-hydroxycholesterol but most likely by 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide, known to be formed by photo-induced air oxidation of cholesterol and demonstrated in these studies to be unresolved from 25-hydroxycholesterol in our thin-layer chromatographic solvent systems. Very complex peroxide mixtures obtain in air-aged cholesterol, as indicated by the many positive peroxidic components detected.

The natural air-aging of cholesterol, whereby the sterol oxidizes, with concomitant lowering of the melting point, yellow coloration, and development of an acrid odor, has fascinated investigators from early times. SCHULZE examined one of his own 25-year old cholesterol samples in 1904 for decomposition, and he recognized at that time the importance of air and light on cholesterol decomposition[48]. A later examination by FIESER with a 24-year old sample[49] implicated several of the autoxidation products detected in the present chromatographic study. Our current reexamination of air-aged cholesterol hinges about a twelve-year old sample of cholesterol of definite history and initially purified to a high degree. At least thirty-two separate sterol components were detected in this sample by our usual two-dimensional chromatographic procedure.

The highly purified twelve-year old sample is free from recognized companion sterols since it was purified *via* the dibromide. It was also free from detectable auto-

xidation products. Accordingly, the several autoxidation products detected in this aged sample must derive from cholesterol *per se*, and not from the usual companion sterols 5α-cholestan-3β-ol, 5α-cholest-7-en-3β-ol, cholesta-5,7-dien-3β-ol, cholest-5-ene-3β,24-diol, etc. of ordinary cholesterol samples. Clearly it is important to establish that all autoxidation products encountered derive from cholesterol rather than from a suspect companion sterol in order to avoid a fruitless search in turn for that companion sterol precursor as in the case of "Ketone 104"[49].

Of the thirty-odd sterol components resolved two-dimensionally, seven have been identified by comparison of their mobilities and color test response to sulfuric acid with those of reference sterols. Thus, cholesta-3,5-dien-7-one, 25-hydroxycholesterol, 7-ketocholesterol, 7β-hydroxycholesterol, 7α-hydroxycholesterol, 3β,5-dihydroxy-5α-cholestan-6-one (previously implicated in cholesterol autoxidation by its isolation from tissues[50]), and 5α-cholestane-3β,5,6β-triol were identified.

Many of these components may be detected in cholesterol samples subjected to heating in air. Indeed, the one-dimensional chromatographic pattern of sterol components produced by mild heating of cholesterol in air is essentially the same as that of naturally air-aged cholesterol, both of the special twelve-year old samples and of aged samples of indefinite history.

In that the oxidized sterol pattern from the twelve-year old sample is more complex than for most shelf-aged samples, it appears that the autoxidation products actually resolved in the shelf-aged samples do derive from the cholesterol component and not likely from minor companion sterol components.

These findings of ready autoxidation of cholesterol under a variety of relatively mild conditions cannot be taken as evidence that autoxidation will occur in every treatment of cholesterol. Suitable schemes for the isolation and analysis of serum cholesterol have been devised, monitored by thin-layer chromatography, and no autoxidation is encountered. Autoxidation does not appear to occur demonstrably on thin-layer chromatography itself despite exposure of the sterol to light and air during usual routine of thin-layer chromatography. Also, although sodium stearate dispersions of cholesterol are autoxidized, albumin dispersions are not. Again, aqueous dispersions prepared by the method of DAY *et al.*[26] are not autoxidized in their preparation or storage. One concludes that the occurrence of autoxidation of cholesterol cannot be predicted with certainty in a given biological system, and careful control work must be done in each instance to assure exclusion of autoxidation as taking place. The thin-layer chromatographic techniques herein outlined offer a simple, rapid means of attaining such control of reactions and isolation procedures.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge support for this work from the National Institutes of Health, U.S. Public Health Service (research grant HE-10160) and from The Petroleum Research Fund, administered by the American Chemical Society.

SUMMARY

The air oxidation of cholesterol has been studied by means of one- and two-dimensional thin-layer chromatographic methods herein developed. A semi-quanti-

tative photoelectric densitometric procedure for evaluation of one-dimensional chromatoplates is also described. The autoxidation of cholesterol by heating or storing in air, irradiation in air, treatment with benzoyl peroxide, purification via the dibromide, and in sodium stearate colloidally dispersed medium has been examined and the autoxidation products resolved by thin-layer chromatography have been catalogued. The complexity of the air oxidation of cholesterol is emphasized as is the necessity of careful control work in chemical and biological systems containing cholesterol.

REFERENCES

1 H. B. MACPHILLAMY, *J. Am. Chem. Soc.*, 62 (1940) 3518.
2 L. RUZICKA AND V. PRELOG, *Helv. Chim. Acta*, 26 (1943) 975.
3 V. PRELOG, E. TAGMANN, S. LIEBERMAN AND L. RUZICKA, *Helv. Chim. Acta*, 30 (1947) 1080.
4 M. HAYANO in O. HAYAISHI (Editor), *Oxygenases*, Academic Press, New York, 1962, p. 190.
5 G. A. D. HASLEWOOD, *Biochem. J.*, 36 (1942) 389.
6 N. L. KANTIENGAR AND R. A. MORTON, *Biochem. J.*, 60 (1955) 25.
7 S. BERGSTRÖM, *Arkiv Kemi, Mineral. Geol.*, 16A (1942) No. 10.
8 S. BERGSTRÖM AND B. SAMUELSSON, in W. O. LUNDBERG (Editor), *Autoxidation and Antioxidants*, Vol. 1, Interscience, John Wiley and Sons, New York, 1961, pp. 233–248.
9 C. W. SHOPPEE, *Ann. Rept. Progr. Chem. (Chem. Soc. London)*, 43 (1947) 215.
10 L. L. SMITH, *J. Am. Chem. Soc.*, 76 (1954) 3232.
11 J. KUČERA, Ž. PROCHÁZKA AND K. VEREŠ, *Collection Czech. Chem. Commun.*, 22 (1957) 1185.
12 L. L. SMITH AND S. J. STATES, *Texas Rept. Biol. Med.*, 12 (1954) 543.
13 A. E. HENDERSON AND J. D. B. MACDOUGALL, *Biochem. J.*, 57 (1954) xxi.
14 C. RIDDELL AND R. P. COOK, *Biochem. J.*, 61 (1955) 657.
15 M. J. D. VAN DAM, G. J. DE KLEUVER AND J. D. DE HEUS, *J. Chromatog.*, 4 (1960) 26.
16 F. NEUWALD AND K.-E. FETTING, *Pharm. Ztg. Ver. Apotheker-Ztg.*, 108 (1963) 1490.
17 J.-R. CLAUDE AND J.-L. BEAUMONT, *Ann. Biol. Clin. (Paris)*, 22 (1964) 815; *J. Chromatog.*, 21 (1966) 189.
18 L. L. SMITH, W. S. MATTHEWS, R. C. BACHMANN AND B. REYNOLDS, *Federation Proc.*, 25 (1966) 770.
19 C. HORVATH, *J. Chromatog.*, 22 (1966) 52.
20 D. WALDI, in E. STAHL (Editor), *Thin-Layer Chromatography, A Laboratory Handbook*, Springer, Berlin, 1965, pp. 483–502.
21 L. F. FIESER, *J. Am. Chem. Soc.*, 75 (1953) 5421; *Organic Syntheses*, Collective Volume 4, John Wiley and Sons, New York, 1963, pp. 195–201.
22 R. NEHER AND A. WETTSTEIN, *Helv. Chim. Acta*, 35 (1952) 276.
23 I. LIFSCHÜTZ AND T. GRETHE, *Ber.*, 47 (1914) 1453.
24 O. WINTERSTEINER AND S. BERGSTRÖM, *J. Biol. Chem.*, 137 (1941) 785.
25 S. BERGSTRÖM AND O. WINTERSTEINER, *J. Biol. Chem.*, 141 (1941) 597.
26 A. J. DAY, N. H. FIDGE AND G. N. WILKINSON, *J. Lipid Res.*, 7 (1966) 132.
27 L. L. SMITH AND T. FOELL, *J. Chromatog.*, 9 (1962) 339.
28 K. SAVARD, *Recent Progr. Hormone Res.*, 9 (1954) 185.
29 K. SCHUBERT, G. ROSE AND M. BÜRGER, *Z. Physiol. Chem.*, 326 (1961) 235.
30 E. H. MOSBACH, M. NIERENBERG AND F. E. KENDALL, *J. Am. Chem. Soc.*, 75 (1953) 2358.
31 R. P. COOK, *Cholesterol, Chemistry, Biochemistry and Pathology*, Academic Press, New York, 1958, p. 494.
32 R. NEHER, *Chromatographie von Sterinen, Steroiden und verwandten Verbindungen*, Elsevier, Amsterdam, 1958, p. 38, p. 69; *J. Chromatog.*, 1 (1958) 122, 160, 205; *Chromatog. Rev.*, 1 (1959) 99; *Steroid Chromatography*, Elsevier, Amsterdam, 1964, p. 73.
33 (a) I. E. BUSH, *The Chromatography of Steroids*, Pergamon, Oxford, 1961, pp. 91–92; (b) Č. MICHALEC, in I. M. HAIS AND K. MACEK (Editors), *Handbuch der Papierchromatographie*, Vol. 1, Gustav Fischer Verlag, Jena, p. 373; in I. M. HAIS AND K. MACEK (Editors), *Paper Chromatography, A Comprehensive Treatise*, Academic Press, New York, 1963, p. 393; Č. MICHALEC AND C. SOBĚSLAVSKÝ, *Chem. Listy*, 53 (1959) 1170.
34 L. RUZICKA, V. PRELOG AND E. TAGMANN, *Helv. Chim. Acta*, 27 (1944) 1149.
35 H. R. B. HUTTON AND G. S. BOYD, *Biochim. Biophys. Acta*, 116 (1966) 336.
36 W. J. MCALEER, M. A. KOZLOWSKI, T. H. STOUDT AND J. M. CHEMERDA, *J. Org. Chem.*, 23 (1958) 958.
37 J. J. PEIFER, *Mikrochim. Acta*, (1962) 529.

38 M. L. BLANK AND O. S. PRIVETT, *J. Lipid Res.*, 4 (1963) 529.
39 O. S. PRIVETT, M. L. BLANK, D. W. CODDING AND E. C. NICKELL, *J. Am. Oil Chemists Soc.*, 42 (1965) 381.
40 A. L. J. BECKWITH, *Proc. Chem. Soc.*, (1962) 194.
41 D. H. R. BARTON AND E. MILLER, *J. Am. Chem. Soc.*, 72 (1950) 1066.
42 I. LIFSCHÜTZ, *Z. Physiol. Chem.*, 106 (1919) 271.
43 G. BLIX AND G. LÖWENHIELM, *Biochem. J.*, 22 (1928) 1313.
44 A. H. THEORELL, *Biochem. Z.*, 175 (1926) 297.
45 T. MOORE AND S. G. WILLIMOTT, *Biochem. J.*, 21 (1927) 585.
46 Y. KASHIO AND J. A. GARDNER, *Biochem. J.*, 24 (1930) 1047.
47 I. M. HAIS AND N. B. MYANT, *Biochem. J.*, 94 (1965) 85.
48 E. SCHULZE AND E. WINTERSTEIN, *Z. Physiol. Chem.*, 43 (1904) 316; 48 (1906) 546.
49 L. F. FIESER, W.-Y. HUANG AND B. K. BHATTACHARYYA, *J. Org. Chem.*, 22 (1957) 1380.
50 E. SCHWENK, N. T. WERTHESSEN AND H. ROSENKRANTZ, *Arch. Biochem. Biophys.*, 37 (1952) 247.