Soc. 78, 377.
Gascoigne, I. M., and Radda, G. K. (1965), *Chem. Commun. 11*, 211.
Gascoigne, I. M., and Radda, G. K. (1967), *Biochim. Biophys. Acta 131*, 498.
Isenberg, I., and Szent-Györgyi, A. (1959), *Proc. Natl. Acad. Sci. U. S. 45*, 1229.
Massey, V. (1963), *Enzymes 7*, 275.
Massey, V., Gibson, Q. H., and Veeger, C. (1961), *in* Free Radicals in Biological Systems, Blois, M. S., Jr., Brown, H. W., Lemmon, R. M., Lindbloom, R. O., and Weissbluth, M., Ed., New York, N. Y., Academic, pp 75–90.
Radda, G. K., and Calvin, M. (1964), *Biochemistry 3*, 384.
Singer, T. P., and Kearney, E. B. (1950), *J. Biol. Chem. 183*, 409.
Suelter, C. H., and Metzler, D. E. (1960), *Biochim. Biophys. Acta 44*, 23.
Wagner, A. E., Walton, E., Boxer, G. E., Pruss, M. P., Holly, F. W., and Folkers, K. (1956), *J. Am. Chem. Soc. 78*, 5079.

Sterol Metabolism. I. 26-Hydroxycholesterol in the Human Aorta*

Johan E. van Lier and Leland L. Smith

ABSTRACT: 26-Hydroxycholesterol has been isolated from healthy and diseased human aortal tissue by means of column, thin layer, and gas chromatographic procedures and identified by comparison of the sterol and its 3β,26-diacetate with authentic samples. Twenty odd sterol-like components present in the polar lipid fraction from the human aorta have been resolved, and 7-ketocholesterol, 25-hydroxycholesterol, 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 5α-cholestane-3β,5,6β-triol have been recognized among the components. 26-Hydroxycholesterol appears to be confined to intimal tissue alone. Gas chromatographic analyses indicate that 26-hydroxycholesterol is present in the human aorta at levels of *ca.* 32 μg/g of dry intimal tissue or 4–100 μg/g of dry combined intimal and medial tissue.

The artifact nature of the 7-hydroxycholesterols and of 25-hydroxycholesterol is suggested, and the role of 26-hydroxycholesterol as a cholesterol companion sterol in the human aorta is considered.

The presence of low levels of other sterols in mammalian tissue cholesterol[1] samples is well recognized. Chief among these minor sterols are cholestanol, 5α-cholest-7-en-3β-ol, cholesta-5,7-dien-3β-ol, cholest-5-ene-3β,24*S*-diol, 7-ketocholesterol, and 5α-cholestane-3β,5,6β-triol. In addition other cholesterol biosynthesis intermediates may be encountered in select cases. Other than sterol biosynthesis intermediates the biological role of the companion sterols is obscure, even for the predominant cholestanol, which has been recognized as a cholesterol companion for 40 years.

Among the tissues most studied for minor sterol composition is the human aorta, with its obvious relation to atherosclerosis. The presence of cholesterol in the diseased human aorta was recognized early (Vogel, 1847). By the early 20th century both cholesterol (Aschoff, 1906; Windaus, 1910; Schoenheimer, 1926, 1928) and cholestanol (Schoenheimer *et al.*, 1930; McArthur, 1942; Mosbach *et al.*, 1963; Kuroda *et al.*, 1964) were established as component sterols in the human aorta.

The levels of cholesteryl fatty acid esters in the human aorta have been extensively reviewed (Tuna and Mangold, 1963). Furthermore, the presence of desmosterol in aortal tissue of patients treated with triparanol has been reported (Blankenhorn *et al.*, 1961; Chobanian and Hollander, 1961, 1965; Jose and Peak, 1963). Other sterols isolated from or detected in the human aorta include cholesta-3,5-dien-7-

* From the Department of Biochemistry, University of Texas Medical Branch, Galveston, Texas 77550. *Received May 22, 1967.* This work was supported by a grant (HE-10160) from the National Institutes of Health of the U. S. Public Health Service. A preliminary report of this work was presented before the meeting of the American Society of Biological Chemists, Chicago, Ill., April 15, 1967 (*Federation Proc. 26*, 342 (1967)).

[1] Systemic nomenclature for steroids given trivial names in the text includes: cholest-5-en-3β-ol, cholesterol; cholest-5-ene-3β,26-diol, 26-hydroxycholesterol; 5α-cholestan-3β-ol, cholestanol; cholesta-5,24-dien-3β-ol, desmosterol; cholest-5-ene-3β,7α-diol, 7α-hydroxycholesterol; cholest-5-ene-3β,7β-diol, 7β-hydroxycholesterol; 3β-hydroxycholest-5-en-7-one, 7-ketocholesterol; cholest-5-ene-3β,24*S*-diol, 24-hydroxycholesterol (cerebrosterol); cholest-5-ene-3β,25-diol, 25-hydroxycholesterol. The 7β-hydroxycholesterol nomenclature used by Hardegger *et al.* (1943), Henderson and MacDougall (1954), and Henderson (1956) appears to refer to the 7α-epimer and has been so altered herein to conform with the correct configurational assignments for these sterols.

one (Hardegger *et al.*, 1943; Kantiengar and Morton, 1955; Robertson, 1955), cholesta-4,6-dien-3-one (Hardegger *et al.*, 1943; Robertson, 1955), 5α-cholestane-3β,5,6β-triol (Hardegger *et al.*, 1943; Henderson and McDougall, 1954; Henderson, 1956), 7α-hydroxycholesterol (Hardegger *et al.*, 1943; Henderson and McDougall, 1954; Henderson, 1956), 7β-hydroxycholesterol (Henderson, 1956), 24- or 25-hydroxycholesterol (Henderson, 1956), and 7-ketocholesterol (Schoenheimer, 1932; Brooks *et al.*, 1966). More recently 26-hydroxycholesterol has been detected as a constituent of human atherosclerotic plaques (Steel *et al.*, 1966; Brooks *et al.*, 1966) and cholesteryl sulfate has been isolated from human aortal tissue (Drayer and Lieberman, 1967).

In the present report we furnish evidence for the presence of 26-hydroxycholesterol as a companion sterol of cholesterol in human aortal tissue, both healthy and diseased.

Experimental Section

Melting points were taken on a calibrated Kofler block under microscope magnification. Infrared absorption spectra were obtained on a Perkin-Elmer Model 337 spectrophotometer equipped with a beam condenser, using pressed potassium bromide disks, either 0.5 or 1.5 mm in diameter, depending on the amount and character of the sample. Ultraviolet absorption spectra were obtained on a Cary Model 14 spectrophotometer using ethanol solutions of sterols.

Thin layer chromatography was conducted on 5 and 20 × 20 cm chromatoplates (0.25 mm thick) prepared with silica gel HF_{254} (E. Merck GmbH, Darmstadt) and on microscope slides coated with silica gel. Irrigation solvent systems included ethyl acetate–heptane (1:1), acetone–heptane (1:1), benzene–ethyl acetate (5:1), and benzene–ethyl acetate (1:1), all conducted in the usual ascending manner in filter paper-lined glass chambers. Resolved components were detected by means of their ultraviolet light absorption properties on the phosphor-coated plates and by their color responses to 50% aqueous sulfuric acid spray. Preparative thin layer chromatography was conducted on 40 and 20 × 20 cm chromatoplates (1 and 2 mm thick) prepared with silica gel HF_{254} or with silica gel PF_{254} (E. Merck). Sterol samples were applied to these plates as chloroform solutions by means of a Rodder Streaker (Rodder Instrument Co., Los Altos, Calif.) so as to deposit 100–200 mg of sample on a 20 × 40 cm chromatoplate 2 mm thick. Irrigation was conducted with the same solvent systems used for analytical thin layer chromatography, with location of resolved components by judicious interpolation from zones detected by spraying a 1-cm portion of the end of the chromatoplate.

Two-dimensional thin layer chromatography was conducted with the same solvent systems as mentioned before (Smith *et al.*, 1967). For the sterol fractions more polar than cholesterol the systems ethyl acetate–heptane (1:1) and acetone–heptane (1:1) were used; for the less polar sterols the systems benzene–ethyl acetate (5:1) and (1:1) were used.

Gas chromatography was conducted on F & M Models 400 and 402 chromatographs using 1.83 m long × 6 mm o.d. glass (silanized) columns packed with either 3% QF-1 (trifluoropropylmethyl silicone) on 100–120 mesh Gas-Chrom Q, 3% SE-30 (methyl silicone) on 80–100 mesh Gas-Chrom Q, or 3% JXR (methyl silicone) on 100–120 mesh Gas-Chrom Q (all from Applied Science Laboratories, State College, Pa.). Injection port temperature was 250° and the columns were operated at 240° with nitrogen carrier gas flow at 22 cc/min. Gas chromatographic mobilities are given in terms of retention times (r_T) relative to cholesterol as unity (retention time of cholesterol: 5.5 min on 3% QF-1, 9.5 min on 3% SE-30, and 7.2 min on 3% JXR).

Preparative gas chromatography was conducted on the same apparatus and columns except that the eluted sterol component was collected in a silanized glass capillary, using the recorder chart drive motor as a timer for the collection as follows. An initial analytical gas chromatogram was run on the sample and the elution curve was recorded in the usual manner. After the final components had eluted, the hydrogen and oxygen supplies for the flame were turned off and the metal flame detector jet was removed and replaced by a Teflon sleeve into which glass capillary tubes could be inserted. The chart paper was turned back to the original point and the forward chart drive was reactivated. As the injection point on the previously drawn chart passed under a fixed reference point on the recorder a second injection of sample (10–100 μg) was made and the chromatogram was run in exactly the same way as was the analytical chromatogram. As the chart position of each component previously recorded passed under the fixed reference point a silanized glass capillary tube (2 × 15 mm) was slipped into the Teflon sleeve and the component was condensed into the capillary within a centimeter or so from the column opening. Components thus isolated were removed from the capillary with methylene chloride (2 μl) for rechromatography on the same and other phases for proof of component integrity, for thin layer chromatography, infrared and ultraviolet absorption measurements, and for melting points. The steryl acetates were easily recrystallized from methylene chloride solutions recovered from the capillaries, but often the free sterols were not. Consequently, melting points on the free sterols were often taken on selected crystals recovered by means of a fine probe from the collection capillary.

Analytical gas chromatography of the isolated sample was necessary to establish the homogeneity of the sample after passage through the gas chromatographic system. Whereas all acetylated sterols were collected without any detectable decomposition, some free sterols could not be so recovered without the appearance of minor more mobile trace components (totals estimated at less than 1%).

Human aortas were taken immediately at autopsy.

Each aorta was washed with distilled water to remove blood clots, pinned to a wax support, and the severity of atheroma was assessed using the recommended classification procedure (W.H.O., 1958). Select portions of the intima and media were dissected from adjoining adventitious tissue. In some cases plaques were dissected separately, so that either two tissue fractions (a combined intima–media sample free from fatty streaks and plaques, stage 0, and a plaque sample at stages I–III) or three tissue fractions (an intimal sample, a medial sample, and a plaque sample) were taken. The samples were cut into small pieces for subsequent extraction and analysis.

Extraction of either aorta sample preparation was accomplished by one of two means. (A) The aorta sample was freeze dried and extracted overnight at −15° under a CO_2 atmosphere with chloroform–methanol (2:1). The extraction was repeated two more times, and the combined chloroform–methanol extracts were evaporated under vacuum in an all-glass rotary vacuum evaporator unit. The yellow oily residue was dissolved in hexane–ether (1:1), filtered, and the solvents were again removed under vacuum. The residue was dissolved in chloroform–methanol (9:1) and stored under CO_2 at −15° pending thin layer chromatographic analysis and column chromatographic fractionation. Additional extraction of the aortal tissue sample afforded a small amount of additional lipid, but the three extractions utilized routinely gave an estimated 98.2% recovery of extractable lipid.

(B) A second extraction method was used for very rapid work. The aorta sample was shredded in a Waring Blendor containing acetone and small pellets of Dry Ice (to cool and to maintain an atmosphere of CO_2). The mixture was filtered and the solids were extracted three times with chloroform–methanol (2:1). The combined lipid extracts were evaporated under vacuum to yield an emulsion of a yellow oil with water. The material was extracted three times with hexane–ether (1:1), the extracts were dried over anhydrous sodium sulfate, filtered, and evaporated under vacuum. The yellow oil was dissolved in chloroform–methanol (9:1) and stored under CO_2 at −15° pending analysis, etc.

The total lipid extract residue was dissolved in hexane and a maximum of 1000 mg of lipid at a time was chromatographed on a 2 × 20 cm column containing 70 g of silica gel (Baker Analyzed reagent grade, activated at 120° for 4 hr). Column fractions of 100 ml were taken with the eluting solvents (200–500 ml of a given solvent mixture): hexane, to elute hydrocarbons; hexane–chloroform (4:1), to elute aldehydes, methyl esters, and steryl esters; hexane–chloroform (1:1), to elute steryl esters; chloroform, to elute sterols less polar than cholesterol and additional chloroform, to elute cholesterol; and chloroform–methanol (10:1), to elute polar sterols. Each eluted fraction was analyzed by thin layer chromatography using the ethyl acetate–heptane (1:1) solvent system, and appropriate column fractions were combined and evaporated under vacuum. Thus were obtained four major lipid fractions of interest: (1) a steryl ester fraction; (2) a minor sterol fraction less polar than cholesterol (containing some steryl esters and cholesterol); (3) a major cholesterol fraction also containing cholestanol, traces of components from the prior fraction 2, and the later fraction 4; and (4) a minor sterol fraction more polar than cholesterol (also containing small amounts of cholesterol and other polar lipid components. A typical two-dimensional thin layer chromatogram of this polar sterol fraction is given in Figure 1, with the several recognized components noted.

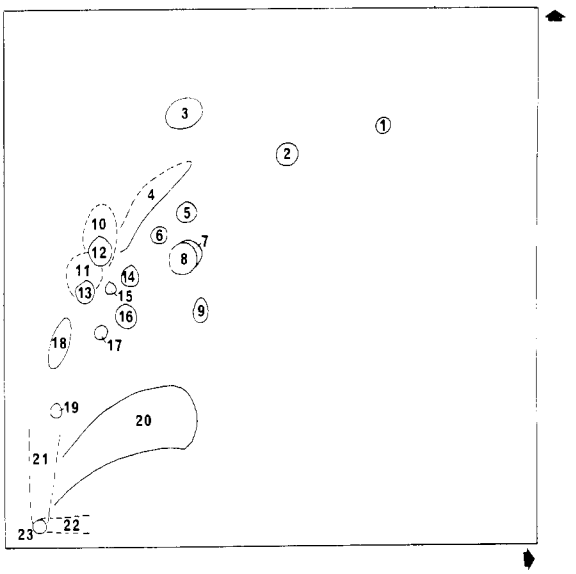

FIGURE 1: Two-dimensional thin layer chromatographic resolution of polar sterol from human aortal tissue (fraction 4) using a slightly deactivated silica gel HF_{254} chromatoplate. Irrigation in the vertical dimension with acetone–heptane (1:1), in the horizontal dimension with ethyl acetate–heptane (1:1). Components identified: (1) cholesta-3,5-dien-7-one, (2) cholesterol, (6) 7-ketocholesterol, (7) 25-hydroxycholesterol, (8) 26-hydroxycholesterol, (14) 7β-hydroxycholesterol, (15) 7α-hydroxycholesterol, and (19) 5α-cholestane-3β,5,6β-triol.

Human liver tissue taken at autopsy was sliced, frozen with Dry Ice, and freeze dried. The freeze-dried tissue was extracted with chloroform–methanol (2:1) according to procedure A.

Human peripheral blood (10-ml samples) obtained as blood bank blood or as freshly drawn whole blood was diluted with an equal volume of distilled water and extracted three times with 100-ml portions of chloroform–methanol (2:1). The extracts were filtered through Celite and anhydrous sodium sulfate and evaporated under vacuum, and the residue was extracted two times with 100 ml of hexane–diethyl ether (1:1), again filtered through Celite and anhydrous sodium sulfate, and concentrated to a small volume under vacuum. The solution was chromatographed

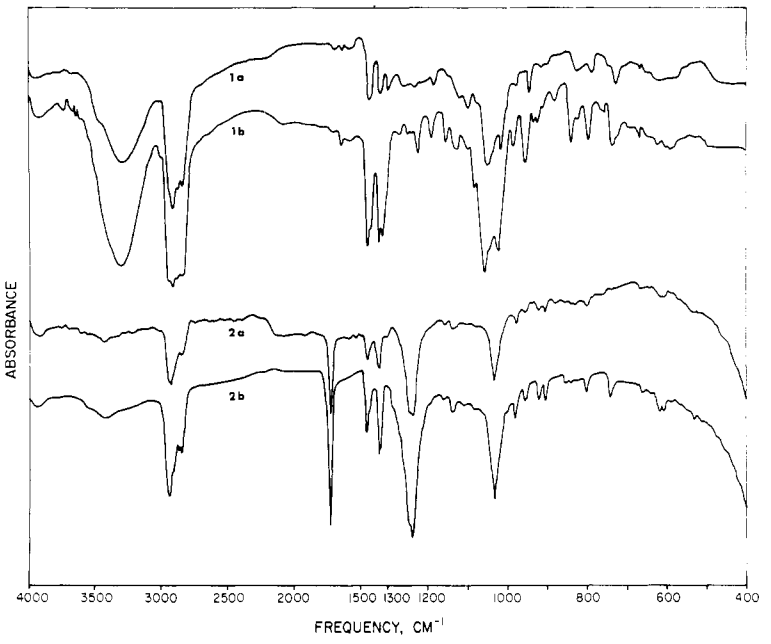

FIGURE 2: Infrared absorption spectral comparisons for aortal 26-hydroxycholesterol and authentic (25*R*)-26-hydroxycholesterol and of their respective 3β,26-diacetates. (1a) Aortal 26-hydroxycholesterol; (1b) authentic 26-hydroxycholesterol; (2a) aortal 26-hydroxycholesterol 3β,26-diacetate; (2b) authentic 26-hydroxycholesterol 3β,26-diacetate.

on 14 g of silica gel, hydrocarbons and esters being eluted with 100 ml of petroleum ether (bp 37–48°)–diethyl ether (9:1), sterols being eluted with 150 ml of diethyl ether–methanol (9:1). The sterol fraction was chromatographed on a 2 mm thick 20 × 20 cm chromatoplate with ethyl acetate–heptane (1:1). The 26-hydroxycholesterol zone was recovered in the usual way, an internal standard of 1 μg of cholesteryl formate was added, and the solution was evaporated under vacuum. The residue was redissolved in 5 μl of chloroform–methanol (9:1) and chromatographed on a 3% SE-30 system using the greatest detector sensitivity possible. No specific response was had at attenuations equivalent to 10 ng/ml of whole blood. Recovery experiments wherein 150 ng of 26-hydroxycholesterol in 200 μl of methanol was added to 10 ml of blood prior to processing established that the over-all procedure gave a 90% recovery at this level (15 ng/ml).

Cholest-5-ene-3β,26-diol. The column minor sterol fraction (4) more polar than cholesterol was dissolved in chloroform and about 100 mg of sample was applied by means of the Rodder Streaker to a 2 mm thick silica gel PF chromatoplate (20 × 40 cm). The chromatogram was irrigated with benzene–ethyl acetate (1:1), and the major sterol zone (located by means of its ultraviolet light absorption properties and by spraying the edge of the chromatogram with 50% sulfuric acid to confirm the identification) was scraped off the chromatoplate and packed into a 2-cm diameter column. Elution of sterols from this column was performed with chloroform–methanol (9:1). The eluates were evaporated under vacuum, and the residue was redissolved in chloroform–methanol (2:1), and stored at −15° under CO_2 pending further analysis. Homogeneity of the eluted sterol was checked by thin layer and gas chromatography. Rechromatography of the sterol on a 1 mm thick 20 × 20 cm silica gel PF chromatoplate (washed chromatographically three times with methanol prior to use) using benzene–ethyl acetate (1:1) gave 26-hydroxycholesterol as the main component. The eluted sterol was dissolved in methylene chloride and held at −10° overnight. The colorless needles which separated were recovered from the mixture by hand using a glass fiber, thus affording crystalline26- hydroxycholesterol, mp 172.5–174.0°, not depressed on admixture with an authentic sample of (25*R*)-26-hydroxycholesterol (lit. (Scheer *et al.*, 1956) mp 177–178 and (Danielsson, 1961) 173–175° for (25*R*)-26-hydroxycholesterol derived from kryptogenin; (Dean and Whitehouse, 1966) mp 168–173° for (25*RS*)-26-hydroxycholesterol). Identity of the 3β,26-diol with the reference sample was further established by thin layer chromatographic behavior, color test response, gas chromatographic behavior (r_T 3.24 on 3% QF-1, 2.25 on 3% SE-30, and 2.48 on 3% JXR), and infrared absorption spectra (Figure 2).

The alternative isolation of 26-hydroxycholesterol from preparative gas chromatography on 3% QF-1 columns (Figure 3) afforded the homogeneous sterol (component 4) (mp 174.5–176.5°) whose infrared absorption spectra, thin layer and gas chromatographic behavior, etc., were identical with those properties of the reference sample. In repeated gas chromatogra-

phic isolations of 26-hydroxycholesterol from different aorta preparations no evidences of inhomogeneity were ever found.

Cholest-5-ene-3β,26-diol 3β,26-Diacetate. A 50-μg sample of aortal 26-hydroxycholesterol dissolved in 500 μl of pyridine and 250 μl of acetic anhydride was held overnight at room temperature, after which time 500 μl of methanol was added, and after 3 hr, 2 ml of water. The mixture was neutralized with aqueous hydrochloric acid and extracted three times with 2 ml of methylene chloride. The combined methylene chloride extracts were washed twice with 1 ml of water and concentrated under vacuum. Final drying was accomplished by addition of toluene and removal of solvent under vacuum. The residue was dissolved in 500 μl of methylene chloride for thin layer and gas chromatographic analysis, which showed a single major sterol component. The methylene chloride solution of the diacetate was gas chromatographed on 3% QF-1 in the same way as used for the free sterol (carrier gas nitrogen flow rate 22 cc/min), and the eluted sterol ester was caught in a glass capillary. The sample was washed from the capillary with 10 μl of methylene chloride. On evaporation of the solvent there was obtained crystals of the 3β,26-diacetate (mp 127.0–128.5°) not depressed (127.0–128.5°) on admixture with the reference 3β,26-diacetate of mp 127.5–128.5° (lit. (Scheer *et al.*, 1956) mp 128–129°). The diacetate was identical with the reference sample as regards thin layer chromatographic behavior, color test response, gas chromatographic characteristics (r_T 7.62 on 3% QF-1, 3.71 on 3% SE-30, and 4.33 on 3% JXR), and infrared absorption spectra (Figure 2).

Cholest-5-ene-3β,25-diol. The preparative gas chromatograms from which 26-hydroxycholesterol was isolated as component 4 (Figure 3) also resolved as a more mobile component a sterol (component 3) which was isolated in a glass capillary in the same manner as described for 26-hydroxycholesterol. The component was washed from the capillary with 5–10 μl of chloroform and the solvent was allowed to evaporate, yielding crystals of 25-hydroxycholesterol (mp 181.0–182.0°) not depressed on admixture with authentic 25-hydroxycholesterol (lit. (Ryer *et al.*, 1950) mp 181.5–182.5, (Dauben and Bradlow, 1950) 177–179, and (Bergmann and Dusza, 1958) 179–180°); ν_{max}^{KBr} 3300, 1050, and 1060 cm^{-1}, identical with spectra of the reference sterol. Identity of the two samples was further established by identical thin layer chromatographic mobilities and color response to 50% sulfuric acid (where a characteristic initial purple-blue coloration develops which rapidly changes to grey-blue) and gas chromatographic retention times (r_T 2.38 on 3% QF-1, 1.56 on 3% SE-30, and 1.78 on 3% JXR).

By very careful work-up of the aortal samples by extraction method A under a carefully maintained atmosphere of CO_2 the amount of 25-hydroxycholesterol (assayed as component 3 of Figure 3) was minimized, and in some preparations none was found.

3β-Acetoxycholest-5-en-25-ol. Mixed aortal sterols (1 mg) recovered from the 26-hydroxycholesterol zone on thin layer chromatograms were acetylated with 1 ml of pyridine and 0.5 ml of acetic anhydride. After standing overnight the solvents were removed under vacuum and the product was processed as described for purification of 26-hydroxycholesterol 3β,26-diacetate. The purified acetylated sterol mixture was dissolved in 0.5 ml of chloroform–methanol (9:1) and injected onto the 3% QF-1 gas chromatographic column. The appropriate steryl acetate elution peak was recovered from the gas chromatogram in the usual manner, from which was obtained crystals of the 3β-monoacetate (mp 138.5–139.5°) not depressed on admixture with the reference steryl 3-monoacetate (lit. (Ryer *et al.*, 1950) mp 142.0–142.8, (Dauben and Bradlow, 1950) 138.5–140.0, and (Bergmann and Dusza, 1958) 139.5–140.5°); ν_{max}^{KBr} 3300, 1250, and 1030 cm^{-1}, identical with spectra of the reference sample. Additionally identical thin layer and gas chromatographic behavior (r_T 4.06 on 3% QF-1, 2.37 on 3% SE-30, and 2.40 on 3% JXR) was exhibited for the two 3β-monoacetate samples.

3β-Hydroxycholest-5-en-7-one. The least mobile component (5 in Figure 3) from the preparative gas chromatogram from which 26-hydroxycholesterol was recovered as component 4 (Figure 3) was isolated in the described manner in a glass capillary. Needles of the sterol were recovered from the capillary (mp 169.5–171.5°) not depressed on admixture with authentic 7-ketocholesterol (lit. (Bergström and Wintersteiner, 1941) mp 170–172, (Prelog *et al.*, 1947) 158–170, (Milburn *et al.*, 1956) 169–170, and (Fieser *et al.*,

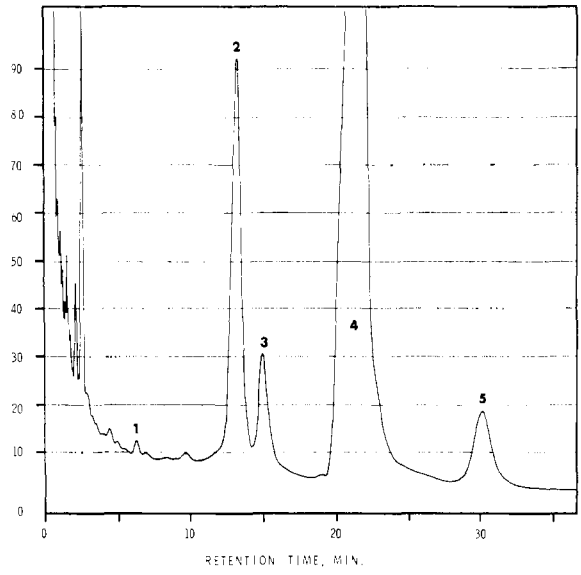

FIGURE 3: Gas chromatographic resolution on 3% QF-1 of polar sterols from the 26-hydroxycholesterol zone of thin layer chromatograms. Component identities: (1) cholesterol, (2) cholesta-3,5-dien-7-one, (3) 25-hydroxycholesterol, (4) 26-hydroxycholesterol, and (5) 7-ketocholesterol.

1957) 171–172°); λ_{max}^{EtOH} 237 nm; ν_{max}^{KBr} 3500, 1650, 1620, and 1060 cm^{-1}, identical with spectra of the reference sterol. Identity was further established by comparison of thin layer chromatographic mobilities, negative color responses to 50% sulfuric acid, and gas chromatographic retention times (r_T 4.70 on 3% QF-1, 1.98 on 3% SE-30, and 2.17 on 3% JXR).

3β-Acetoxycholest-5-en-7-one. The appropriate elution peak from the acetylated polar sterol was recovered from the gas chromatogram as hexagonal crystals from chloroform, with a dramatic phase transition change at 150° to fine needles (mp 157–158°) not depressed on admixture with authentic 7-ketocholesteryl acetate (lit. (Bergström and Wintersteiner, 1941) 157–159, (Prelog *et al.*, 1947) 154–156, (Milburn *et al.*, 1956) 155, and (Fieser *et al.*, 1957) 158–160°);λ_{max}^{EtOH} 237 nm; ν_{max}^{KBr} 1720, 1650, 1620, 1265, and 1040 cm^{-1}, identical with spectra of the authentic steryl acetate. Identity was also established by comparison of thin layer and gas chromatographic behavior (r_T 7.05 on 3% QF-1, 2.50 on 3% SE-30, and 2.70 on 3% JXR).

Cholesta-3,5-dien-7-one. The most mobile component (2) after cholesterol on the preparative 3% QF-1 column from which 26-hydroxycholesterol (4) was recovered was collected on elution. Rechromatography on 3% SE-30 and recovery of the major component afforded cholesta-3,5-dien-7-one (λ_{max}^{EtOH} 280 nm; ν_{max}^{KBr} 1650, 1620, 1590, and 880 cm^{-1}, identical with spectra of an authentic sample). Identity was further established by comparison of thin layer chromatographic mobilities, color response to 50% sulfuric acid (beige color), and gas chromatographic behavior (r_T 2.14 on 3% QF-1, 1.25 on 3% SE-30, and 1.33 on 3% JXR). The cholesta-3,5-dien-7-one preparation was shown by gas chromatographic analyses to be contaminated with traces of other unidentified sterols, of which one was a major component.

Quantitative Determinations. The relative levels of 26-hydroxycholesterol present in several lipid samples were determined on sterol preparations after initial thin layer chromatography, location of the 26-hydroxycholesterol zone by comparison with reference 26-hydroxycholesterol run at the same time, on the same chromatoplate, and elution of the sterol with chloroform–methanol (9:1). The chloroform–methanol extract was evaporated under vacuum, the residue was dissolved in 100–500 μl of chloroform–methanol (9:1), and 2–5-μl aliquots, containing 0.1–0.5 μg of 26-hydroxycholesterol were injected into the 3% QF-1 analytical column operated at 240°. Peak area for the resolved sterol components was measured by weighing the paper under the elution curve peak of interest and comparison against a calibration curve constructed with 26-hydroxycholesterol. The calibration curve for reference 26-hydroxycholesterol was linear over the range examined (70–550 ng).

Remarks

The presence of many unidentified sterol-like components in human aortal lipids is clearly demonstrated in our current studies. In the face of the component complexity encountered in the total lipid extracts and in partially purified preparations, our preliminary attempts at cataloging the resolved sterols in anticipation of their systemic identification have been limited to a specific polar sterol zone defined chromatographically in the Experimental Section. However, within the broad recognition of components, cholesterol, cholesteryl esters, and several more polar sterols whose color response to sulfuric acid was characteristic have been recognized in the total lipid extracts.

Isolation of the select polar sterol fraction required preliminary separation from the large amounts of cholesterol and esters, and during these steps the added complication of artifact formation was obtained. In view of the facile air oxidation of cholesterol handled under the usual laboratory conditions (Hais and Myant, 1965; Smith *et al.*, 1967) substantial efforts were made to avoid artifact formation or to limit its extent. Despite such measures artifacts were discovered in several samples, thus necessitating great care and additional work in order to differentiate among the naturally occurring polar sterols and the artifact sterols in a given sample.

A catalog of 20 odd sterol-like components from a typical polar sterol fraction of the human aorta may be constructed from the two-dimensional thin layer chromatogram of Figure 1. Cholesterol, the epimeric 7-hydroxycholesterols, and 5α-cholestane-3β,5,6β-triol were recognized by inspection of their color responses, and 7-ketocholesterol by its strong ultraviolet light absorption, lack of color response, and reduction to 7-hydroxycholesterol (Smith and Price, 1967). These same sterols were also regularly detected in the unfractionated aortal total lipid fraction.

A new sterol (component 8) giving the characteristic magenta coloration of cholesterol, present in both unfractionated aortal lipids and in the isolated polar sterol fraction, received our early attention. Although repetitive thin layer chromatographic procedures gave some added purification of the sterol, the complexity of the sterol (component 8) zone was readily recognized by gas chromatography, where typically a five-component elution curve was obtained (Figure 3). We have previously noted the complexity of this sterol region on our two-dimensional thin layer chromatoplates, on which are found the several sterols 7-ketocholesterol, 25-hydroxycholesterol, cholest-5-ene-3β,4β-diol, and 3β-hydroxy-5α-cholest-6-en-5-hydroperoxide, all implicated in cholesterol autoxidation (Smith *et al.*, 1967).

The most mobile component in the gas chromatographic resolution (peak 1, Figure 3) was recognized as residual cholesterol. The amount of this component varied widely, depending on the detailed prior handling of the polar sterol fraction. In those cases where very narrow bands of polar sterol were cut from preparative one-dimensional chromatoplates it was possible to reduce the amount of cholesterol to undetectable levels.

Identification of the major sterol component (peak 4, Figure 3) as 26-hydroxycholesterol, and of the minor components from peak 3 as 25-hydroxycholesterol, peak 5 as 7-ketocholesterol, and peak 2 as cholesta-3,5-dien-7-one is detailed in the Experimental Section. Rigorous identification of 26-hydroxycholesterol and of the several other sterols was made on several aortal samples by means of the preparative gas chromatographic methods already described, so that a high degree of confidence in these identifications was attained.

The presence of these polar sterols in the original aortal tissue must be carefully considered since, with the exception of 26-hydroxycholesterol, each figures prominently in the autoxidation of cholesterol under a variety of conditions. In addition the stability of each sterol toward the chromatographic procedures utilized requires evaluation.

The integrity of 26-hydroxycholesterol throughout our procedures was readily demonstrated. Only very minor decomposition was detected for 25-hydroxycholesterol. By very rapid processing of aorta tissue samples at low temperature, using great care to exclude all traces of air and light, it was not possible to detect 25-hydroxycholesterol in the polar sterol fraction. The variable presence of this sterol depended on the isolation procedures employed and on the nature of exposure of the sterol sample to light and air. It was possible to observe qualitatively the increase in 25-hydroxycholesterol in samples treated less carefully.

The recognized nature of 25-hydroxycholesterol as an autoxidation artifact of solid cholesterol (Fieser *et al.*, 1957; Beckwith, 1962; Matthews and Smith, 1966; Smith *et al.*, 1967) has not generally been extended to tissue studies. Since we have been unable to demonstrate the presence of 24*S*-hydroxycholesterol (Ercoli and de Ruggieri, 1953; Ercoli *et al.*, 1953) (which is resolved in our systems and which would be detected if present at levels above 1–10 ng/g of dry tissue) in human aortal polar sterols, we consider the tentative recognition of 24- or 25-hydroxycholesterol in the human aorta by Henderson (1956) may actually have dealt with 25- or 26-hydroxycholesterol. 25-Hydroxycholesterol has also been detected in rat liver mitochondrial incubations of cholesterol along with 26-hydroxycholesterol (Frederickson and Ono, 1956; Kritchevsky and Tepper, 1966), but the presence of 25-hydroxycholesterol in such preparations was not confirmed by Danielsson (1961). Finally, some evidence has been presented for the presence of 25-hydroxycholesterol in human umbilical cord blood (Eberlein and Patti, 1965).

These several points lead us to conclude that 25-hydroxycholesterol occurs in our aortal preparations as an artifact of air oxidation rather than as a normal constituent, and that 25-hydroxycholesterol may be encountered in other tissue sterols as an artifact.

The question of 7-ketocholesterol as a normal constituent of the aorta requires other consideration. 7-Ketocholesterol has been detected or isolated as such or as its dehydration product cholest-3,5-dien-7-one from a variety of tissues, including the human aorta (Schoenheimer, 1932; Hardegger *et al.*, 1943; Kantiengar and Morton, 1955; Brooks *et al.*, 1966). However, the extent of air oxidation as a contributory process was either not considered by these investigators or else was rejected as a factor (Kantiengar and Morton, 1955). No prior effort is known to us which offers the careful chromatographic or other control work and attention to detail necessary to definitive conclusions on the matter.

7-Ketocholesterol was detected in widely variable amounts in every 26-hydroxycholesterol preparation from human aortal tissue which we examined. In no instance did we find 26-hydroxycholesterol free from 7-ketocholesterol as a contaminant or companion sterol. The levels of 7-ketocholesterol exceeded those of the recognized autoxidation artifact 25-hydroxycholesterol but were always less than those of 26-hydroxycholesterol. Whereas the appearance of and the increase in the amount of 25-hydroxycholesterol and the 7-hydroxycholesterol epimers were under our direct observation during the analyses described, such was not the case for 7-ketocholesterol. Although these factors are not conclusive we presently consider that the 7-ketocholesterol encountered in human aortal tissue was not generated by our analytical and isolation techniques.

The presence of cholesta-3,5-dien-7-one (as peak 2, Figure 3) in the 26-hydroxycholesterol fraction must be ascribed to decomposition of the 7-ketocholesterol therein present, since prior adsorption chromatography excluded cholesta-3,5-dien-7-one which may have been present initially in the total sterol fraction. 7-Ketocholesterol appears to be dehydrated on our thin layer chromatographic elution and recovery procedures and also on injection into the flash heater zone of the gas chromatographic columns. Recovery of 7-ketocholesterol in capillaries after elution from the column also tends to give some contaminating cholesta-3,5-dien-7-one. Recovery of both 7-ketocholesterol and cholesta-3,5-dien-7-one after gas chromatography required careful attention, since the 7-ketocholesterol contained some dehydration product and the cholesta-3,5-dien-7-one fraction from the sterol mixture appears to be contaminated by an as yet unidentified sterol component.

It must be emphasized that our detection of cholesta-3,5-dien-7-one in the present sterol fractions neither confirms nor denies the prior claims for its isolation from or detection in the human aorta (Hardegger *et al.*, 1943; Kantiengar and Morton, 1955; Robertson, 1955), since our results apply here only to aortal polar sterol fractions initially more polar than cholesterol on column and thin layer chromatoplates. The ready dehydration of 7-ketocholesterol herein observed in relatively uncomplicated chromatographic procedures does support an artifact status for cholesta-3,5-dien-7-one recovered from tissues where controlled chromatographic observation of the several steps involved was not had.

Accordingly we account for the five-component

sterol mixture (Figure 3) from the aortal polar lipid fraction as: component 1, cholesterol, as a polar sterol fraction contaminant derived from the large amount of cholesterol present in the tissue initially; component 2, cholesta-3,5-dien-7-one, formed from 7-ketocholesterol on elution from thin layer chromatograms and from preparative gas chromatography, therefore an artifact of isolation; component 3, 25-hydroxycholesterol, presumed to be an artifact of isolation and not present in tissue; component 4, 26-hydroxycholesterol, a true companion sterol; and component 5, 7-ketocholesterol, probably a companion sterol rather than artifact.

Other polar sterols detected in the polar sterol fraction but not isolated and identified by rigorous techniques include the epimeric 7-hydroxycholesterols and probably 5α-cholestane-3β,5,6β-triol, together with several unidentified very polar components (Figure 1). As with 25-hydroxycholesterol isolated from the 26-hydroxycholesterol zone of the thin layer chromatogram, the epimeric 7-hydrocholesterol components were not always encountered, their levels were variable, and the proportions of the 7α- and 7β-epimers were variable, in such a manner as to suggest an artifact origin. The co-occurrence of the epimeric 7-hydroxycholesterols, 7-ketocholesterol, and 5α-cholestane-3β,5,6β-triol in sterol preparations strongly suggests autoxidation, and although Danielsson (Danielsson, 1960; Danielsson and Einarsson, 1964) has devised means of demonstration of enzymatic 7α-hydroxylation in the presence of autoxidative attack, our own static isolation experiments are not directly adapted to these approaches. Accordingly, we do not offer definitive argument for the ultimate origin of these sterols in this report.

Discussion

The present finding of 26-hydroxycholesterol and 7-ketocholesterol in the human intima, together with those reports of Tuna and Mangold (1963), Steel *et al.* (1966), and Brooks *et al.* (1966), support the thesis that low levels of companion sterols do occur in the human aorta. Other recent work (Kaufmann and Viswanathan, 1964; Chobanian and Hollander, 1965) using similar chromatographic procedures was not directed to the question of companion sterols. As demonstrated herein, the serious problem of artifact encounter during analysis must be satisfactorily overcome in all such work lest conclusions be compromised. Although Haslewood (1944) initially raised such doubts in connection with the work of Hardegger *et al.* (1943) and Böttcher (1963) has recently reiterated the viewpoint, the specific case of 26-hydroxycholesterol appears to warrant consideration as an enzymatic product rather than as an artifact. To date 26-hydroxycholesterol has not been implicated in sterol autoxidation, nor is there a reasonable basis for postulating it as a result of autoxidation of cholesterol.

Although cholesterol biosynthesis in the mammalian aorta has been demonstrated under select conditions

TABLE I: 26-Hydroxycholesterol Levels in the Human Aorta.

No.	Sample	Freeze-Dried Tissue (g)	Total Lipids		Cholesterol			26-Hydroxycholesterol		
			mg	%	mg	mg/g of Tissue	% of Lipids	μg	μg/g of Tissue	μg/g of Lipids
1	Pooled media, intima, and plaques of five adult aortas, stages 0–II	20.908	2842	13.6	400	19.1	14.1	300	14.4	105
2	Media and intima of adult aorta, stage 0–I	1.393	169	12.1	30	21.5	17.7	140	100	828
3	Ulcerated, calcified plaques, stage III from same individual as 2	1.676	451	26.9	90	53.7	20.0	22	13.1	48.8
4	Media and intima, young child, stage 0	0.651	24.3	3.7				2.5	3.8	103
5	Pooled intima, six adult aortas, stages I–III	13.6	1816	13.4				441	32.4	243
6	Pooled medias, six adult aortas from same source as 5	15.3	724	4.7				0	0	0

and very recently cholestanol biosynthesis in the human intima was established (Chobanian, 1967), biosynthesis of other sterols by intimal tissue has not been suitably examined. In the absence of a demonstrated 26-hydroxylase system in the human intima the biosynthesis origin of intimal 26-hydroxycholesterol is obscure, though subject to speculation. Mouse and rat liver has been shown to contain a 26-hydroxylase which utilizes cholesterol (Fredrickson, 1956; Fredrickson and Ono, 1956; Danielsson, 1961; Berséus, 1965), desmosterol (Danielsson and Johansson, 1964), and 7α-hydroxycholest-4-en-3-one (Hutton and Boyd, 1966) as substrate, and a concept of an hepatic origin with plasma transport and imbibition in the intima of 26-hydroxycholesterol requires consideration. However, sterol 26-hydroxylase systems have not been described for human liver, and we have been unable to demonstrate the presence of 26-hydroxycholesterol in either human peripheral blood or liver to our satisfaction. Although gas chromatographic peaks have been obtained at retention times suitable for 26-hydroxycholesterol, independent identification of the sterol has not been possible, and the low levels (less than 10 ng/ml of whole blood, less than about 1–10 ng/g dry liver) leave this aspect unsettled.

The concentration of 26-hydroxycholesterol in the intima, with little or none in the media (Table I), both healthy and diseased, and the demonstrated biosynthesis activity of the intima in respect to cholestanol raises the provocative question of the significance of these two sterols in respect to atherosclerosis, particularly when it is recalled that both manifest toxic properties in chick heart and rabbit aorta preparations (Biswas *et al.*, 1964; MacDougall *et al.*, 1965a,b).

Acknowledgments

The authors wish to thank Dr. F. L. Jennings and associates of the Department of Pathology of this University for their aid in obtaining human autopsy material. Appreciation is also expressed to Professor L. F. Fieser, Harvard University, for a sample of 24-hydroxycholesterol; Dr. G. Steel, Medical Research Council, Glasgow, and to Mr. J. R. Mitton, University of Edinburgh Medical School, for generous samples of (25*R*)-26-hydroxycholesterol.

References

Aschoff, L. (1960), *Verhandl. Deut. Ges. Pathol. 10*, 166.

Beckwith, A. L. J. (1962), *Proc. Chem. Soc.*, 194.

Bergmann, W., and Dusza, J. P. (1958), *J. Org. Chem. 23*, 459.

Bergström, S., and Wintersteiner, O. (1941), *J. Biol. Chem. 141*, 597.

Berséus, O. (1965), *Acta Chem. Scand. 19*, 325.

Biswas, S., MacDougall, J. D. B., and Cook, R. P. (1964), *Brit. J. Exptl. Pathol. 45*, 13.

Blankenhorn, D. H., Maronde, R. F., and Scholtz, J. R. (1961), *Circulation 24*, 889.

Böttcher, C. J. F. (1963), *in* Evolution of the Atherosclerotic Plaque, Jones, R. J., Ed., Chicago, Ill., University of Chicago, p 138.

Brooks, C. J. W., Harland, W. A., and Steel, G. (1966), *Biochim. Biophys. Acta 125*, 620.

Chobanian, A. V. (1967), *Federation Proc. 26*, 262.

Chobanian, A. V., and Hollander, W. (1961), *Circulation 24*, 1086.

Chobanian, A. V., and Hollander, W. (1965), *J. Lipid Res. 6*, 37.

Danielsson, H. (1960), *Acta Chem. Scand. 14*, 846.

Danielsson, H. (1961), *Arkiv Kemi 17*, 373.

Danielsson, H., and Einarsson, K. (1964), *Acta Chem. Scand. 18*, 831.

Danielsson, H., and Johansson, G. (1964), *Acta Chem. Scand. 18*, 788.

Dauben, W. G., and Bradlow, H. L. (1950), *J. Am. Chem. Soc. 72*, 4248.

Dean, P. D. G., and Whitehouse, M. W. (1966), *Biochem. J. 98*, 410.

Drayer, N. M., and Lieberman, S. (1967), *J. Clin. Endocrinol. Metab. 27*, 136.

Eberlein, W. R., and Patti, A. A. (1965), *J. Clin. Endocrinol. Metab. 25*, 1101.

Ercoli, A., and de Ruggieri, P. (1953), *J. Am. Chem. Soc. 75*, 3284.

Ercoli, A., Di Frisco, S., and de Ruggieri, P. (1953), *Gazz. Chim. Ital. 83*, 78.

Fieser, L. F., Huang, W.-Y., and Bhattacharyya, B. K. (1957), *J. Org. Chem. 22*, 1380.

Fredrickson, D. S. (1956), *J. Biol. Chem. 222*, 109.

Fredrickson, D. S., and Ono, K. (1956), *Biochim. Biophys. Acta 22*, 183.

Hais, I. M., and Myant, N. B. (1965), *Biochem. J. 94*, 85.

Hardegger, E., Ruzicka, L., and Tagmann, E. (1943), *Helv. Chim. Acta 26*, 2205.

Haslewood, G. A. D. (1944), *Nature 154*, 29.

Henderson, A. E. (1956), *J. Histochem. Cytochem. 4*, 153.

Henderson, A. E., and MacDougall, J. D. B. (1954), *Biochem. J. 57*, xxi.

Hutton, H. R. B., and Boyd, G. S. (1966), *Biochim. Biophys. Acta 116*, 362.

Jose, A. D., and Peak, H. J. (1963), *Brit. Heart J. 25*, 133.

Kantiengar, N. L., and Morton, R. A. (1955), *Biochem. J. 60*, 25.

Kaufmann, H. P., and Viswanathan, C. V. (1964), *Fette Seifen Anstrichmittel 65*, 839.

Kritchevsky, D., and Tepper, S. A. (1966), *Experientia 22*, 84.

Kuroda, M., Werbin, H., and Chaikoff, I. L. (1964), *Anal. Biochem. 9*, 75.

MacDougall, J. D. B., Biswas, S., and Cook, R. P. (1965a), *Brit. J. Exptl. Pathol. 46*, 549.

MacDougall, J. D. B., Biswas, S., and Cook, R. P. (1965b), *J. Anat. 99*, 211.

Matthews, W. S., and Smith, L. L. (1966), *Texas Rept. Biol. Med. 24*, 515.

McArthur, C. S. (1942), *Biochem. J. 36*, 559.

Milburn, A. H., Truter, E. V., and Woodford, F. P. (1956), *J. Chem. Soc.*, 1740.
Mosbach, E. H., Blum, J., Arroyo, E., and Milch, S. (1963), *Anal. Biochem. 5*, 158.
Prelog, V., Tagmann, E., Lieberman, S., and Ruzicka, L. (1947), *Helv. Chim. Acta 30*, 1080.
Robertson, D. M. (1955), *Biochem. J. 61*, 681.
Ryer, A. I., Gebert, W. H., and Murrill, N. M. (1950), *J. Am. Chem. Soc. 72*, 4247.
Scheer, I., Thompson, M. J., and Mosettig, E. (1956), *J. Am. Chem. Soc. 78*, 4733.
Schoenheimer, R. (1926), *Z. Physiol. Chem. 160*, 61.
Schoenheimer, R. (1928), *Z. Physiol. Chem. 177*, 143.
Schoenheimer, R. (1932), *Z. Physiol. Chem. 211*, 65.
Schoenheimer, R., von Bering, H., and Hummel, R. (1930), *Z. Physiol. Chem. 192*, 93.
Smith, L. L., Matthews, W. S., Price, J. C., Bachmann, R. C., and Reynolds, B. (1967), *J. Chromatog. 27*, 187.
Smith, L. L., and Price, J. C. (1967), *J. Chromatog. 26*, 509.
Steel, G., Brooks, C. J. W., and Harland, W. A. (1966), *Biochem. J. 99*, 51P.
Tuna, N., and Mangold, H. K. (1963), *in* Evolution of the Atherosclerotic Plaque, Jones, R. J., Ed., University of Chicago, Chicago, Ill., p 85.
Vogel, J. (1847), The Pathological Anatomy of the Human Body, Philadelphia, Pa., Lea and Blanchard, pp 350–351, 531.
W.H.O. (1958), Classification of Atherosclerotic Lesions, Technical Report Series, No. 143, p 4.
Windaus, A. (1910), *Z. Physiol. Chem. 67*, 174.