Accumulation of oxygenated steryl esters in Wolman's disease

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Abstract 7α- and 7β-hydroxycholesteryl esters, 7-ketocholesteryl esters, and 5,6α- and 5,6β-epoxycholesteryl esters have been identified in tissues of patients affected by Wolman's disease. Their structural identities were determined by mass spectroscopy and nuclear magnetic resonance spectroscopy and confirmed by chemical synthesis. It is postulated that cholesteryl ester hydrolase deficiency in Wolman's disease might lead to accumulation of oxygenated steryl esters in vivo and impairment of bile acid formation.

Supplementary key words 7α-hydroxycholesteryl esters · bile acid precursors · cholesteryl ester hydrolase

Wolman's disease is a rare familial disorder characterized by bilateral adrenal calcification, intestinal symptoms (steatorrhea), hepatosplenomegaly, and a failure to thrive. The disorder becomes clinically evident in the first few weeks of life and is invariably fatal, usually by the age of 6 months (1–3).

The nature of the biochemical defect is not completely understood. It has been known for some time that cholesteryl esters and triglycerides accumulate in liver, spleen, and other organs of such patients. More recently, it has been suggested that the deficiency of a lysosomal acid lipase acting on both triglycerides and cholesteryl esters leads to the progressive deposition of these lipids in affected tissues (4). An acid lipase deficiency has also been demonstrated, using tridecanoate as substrate, in cultured fibroblasts (5). To date, however, it is not certain if the accumulation of triglycerides and cholesteryl esters results from the deficiency of a single acid lipase. Purification of the enzyme is required to ascertain its substrate specificity.

We have recently examined the neutral lipid composition of several organs of two patients with Wolman's disease and have found a number of compounds to be present that have not been described in previous analyses. These compounds and some of their chemical derivatives were characterized by gas–liquid chromatography, mass spectrometry, and nuclear magnetic resonance spectroscopy and were found to be oxygenated steryl esters. Their identities were confirmed by chemical synthesis.

By the qualitative techniques employed, none of these esters were found in tissues obtained from controls, including patients with two other disorders associated with cholesteryl ester accumulation (Tangier disease and cholesteryl ester storage disease). It is speculated that Wolman's disease may be associated with impairment of bile acid formation due to the cholesteryl ester hydrolase deficiency.

MATERIALS AND METHODS

Clinical aspects

(*a*) *Wolman's disease.* Tissues were obtained from two patients with Wolman's disease (D.D. and A.J.). Those from D.D. were kindly supplied by Dr. Allen Crocker, Children's Medical Center, Boston. The clinical course of this patient has been described (6); she had a sibling who also died of the same disorder. Material from patient A.J. (Buffalo Children's Hospital, no. 01-30-83) was kindly supplied by Dr. J. Courtner. This patient had a clinical course quite similar to that of D.D., except that she experienced little vomiting and diarrhea. Abdominal distention was first noted at 8 wk of age and was associated with massive hepatosplenomegaly. X-rays of the abdomen revealed large, calcified adrenal glands. Her condition progressively deteriorated; she developed severe anemia, thrombocytopenia, and marked jaundice and expired at 11 wk of age in cardiorespiratory arrest. The tissues of patient D.D. and A.J. were obtained 4 hr after death and

Abbreviations: GLC, gas–liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; TMSi, trimethylsilyl.

TABLE 1. Tissue lipid concentration

Patient	Disease	Liver			Spleen		
		Cholesteryl Ester	Free Cholesterol	Glycerides	Cholesteryl Ester	Free Cholesterol	Glycerides
		mg/g wet wt			*mg/g wet wt*		
L.Mc.	CESD[a]	94.6	5.2	33.4	1.9	0.5	32.5
A.J.	Wolman's	24.3	9.8	42.1	3.4	0.3	3.0
D.D.	Wolman's	28.0	14.0	44	3.9	0.3	3.5
Normal[b]		1.0 ± 0.3	1.5 ± 0.4	19.4 ± 5.2	0.5 ± 0.2	<0.5	<0.5

[a] Cholesteryl ester storage disease.
[b] Mean values for normal tissue from three young adults.

were stored at −20°C. Tissues of patient D.D. had been stored for 11 yr and those from A.J. for 20 months prior to analyses. The concentrations of cholesterol, cholesteryl esters, and glycerides in tissues from the two patients are provided in **Table 1.**

(*b*) *Cholesteryl ester storage disease.* Patient L.Mc. was the first known example of this disease, and her clinical history and pathological findings have been extensively described (3, 7, 8). The patient expired in heart failure at 21 yr of age; she had a severely stenotic aortic valve. Tissues from L.Mc. were obtained 5 hr after her death and were stored at −20°C for 15 months prior to analyses. Lipid analyses of tissues from L.Mc. are shown in Table 1 and have been described elsewhere (3).

(*c*) *Tangier disease.* Tonsils were obtained from E.L. and T.L. and the spleen from C.N., three patients homozygous for Tangier disease, and tonsils were obtained from H.M., a presumed heterozygote for Tangier disease. These patients have been previously described in detail (9–12).

(*d*) *Other tissues.* Control tonsils were frozen immediately after removal for benign hypertrophy at Children's Hospital, Washington, D.C. Portions were analyzed previously (9, 11), and the rest was retained at −20°C for 13 yr before further analysis in this study. Three pairs of adrenals as well as pieces of liver from young adults were obtained during autopsy after death unrelated to lipid storage disease. These tissues were kept frozen at −20°C between 2 and 24 months prior to analyses.

Tissue lipid extractions

Lipids were extracted from 0.5- to 2-g samples of tissue by homogenizing them for 2 min at 4°C with 40 ml of chloroform–methanol 2:1 in a Sorvall Omni-Mixer. The residue was removed by filtration, and the filter cake was washed with an additional 50 ml of chloroform–methanol 2:1. The lipid extract was then partitioned and washed as described by Folch, Lees, and Sloane Stanley (13).

Neutral lipids were separated from the lower phase by silicic acid column chromatography. 100 mg of lipid was applied to a 25-g silicic acid column. Glycerides, sterols, and steryl esters were completely eluted from the column with 250 ml of chloroform–methanol 99:1. They were separated by TLC, using petroleum ether–ether–acetic acid 90:10:1 as solvent system, separately scraped from the plate, and eluted with chloroform–methanol 2:1. The cholesterol content was measured in a Technicon AutoAnalyzer by the ferric chloride method (14); glycerides were measured by the fluorometric method of Kessler and Lederer (15).

Reference compounds

5-Cholestene-3β,7α-diol (mp 180–182°C), 5-cholestene-3β,7β-diol (mp 177–179°C), 5-cholesten-3β-ol-7-one (mp 165–167°C), cholestane-5α,6α-epoxy-3β-ol (mp 143–144°C), and cholestane-3β,5α,6β-triol triacetate (mp 150–151°C) were obtained from Steraloids, Inc.

Cholestane-5β,6β-epoxide (mp 131°C) was synthesized from cholestane-3β,5α,6β-triol triacetate by the method of Davis and Petrow (16). Oleoyl esters of cholesterol and oxygenated cholesterols were prepared according to the method of Deykin and Goodman (17). The esters were purified by repeated preparative TLC (solvent system: petroleum ether–diethyl ether–acetic acid 90:10:1). Homogeneity of the pure compounds was verified by analytical TLC.

Chromatographic and spectroscopic methods

One-dimensional TLC was performed on plates of silica gel G. The nonpolar solvent system used for separation of neutral lipids was petroleum ether–diethyl ether–acetic acid 90:10:1 (18). Reference steroids were detected by spraying the chromatograms with sulfuric acid (19) or as zones made fluorescent by spraying with 8-hydroxy-1,3-pyrenetrisulfonic acid sodium salt, 5 mg in 100 ml of methanol, and exposing to long-wave UV light (20).

GLC was carried out on a Barber-Colman model 5000 gas chromatograph equipped with hydrogen flame detector. Separations of neutral steroids were made on 2-m silanized glass tubes packed with 3% OV-210 phases on 100–120 mesh Supelcoport (Supelco, Inc., Bellafonte, Pa.) (injection port temperature, 260°C; column temperature, 240°C; detector temperature, 270°C). Fatty acid methyl esters were analyzed on a 4-ft column packed with 15%

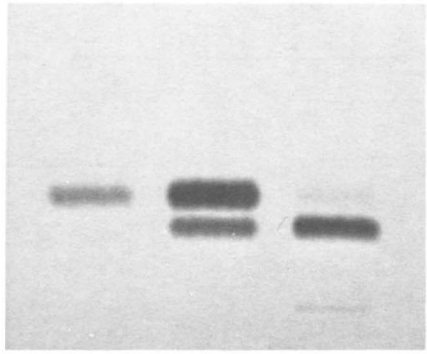

Fig. 1. Thin-layer chromatogram of 7α-hydroxycholesteryl oleate and 7β-hydroxycholesteryl oleate. An isomeric mixture of the two was prepared by $NaBH_4$ reduction of 7-ketocholesteryl oleate (see Materials and Methods) and separated by column chromatography and preparative TLC. *Center,* isomeric mixture; *left,* purified 7β-hydroxycholesteryl oleate; *right,* prepurified 7α-hydroxycholesteryl oleate. Solvent system, petroleum ether–ether–acetic acid 90:10:1. In this and all subsequent TLC photographs, spots were obtained by charring in the presence of 50% H_2SO_4.

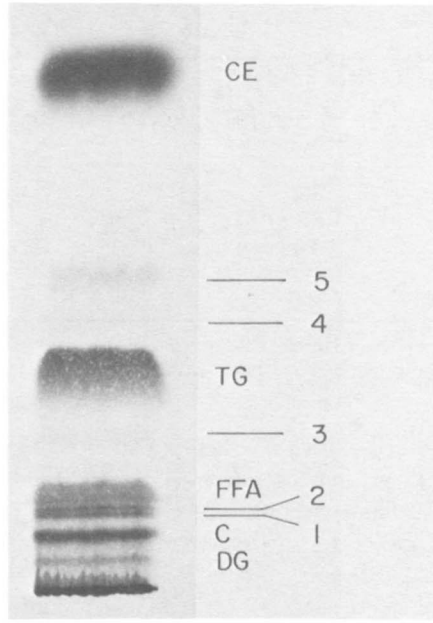

Fig. 2. Thin-layer chromatogram of total lipid extract of liver from patient D.D. with Wolman's disease. CE, cholesteryl ester; TG, triglycerides; DG, diglycerides; FFA, free fatty acids; C, cholesterol; unknown lipids, fractions 1–5.

diethylene glycol succinate polyester on 60–70 mesh Anakrom A and operated at 180°C.

NMR spectra were observed on a Varian XL-100-15 spectrometer in chloroform solution. Chemical shifts are reported in parts per million to tetramethylsilane ($\delta = 0$).

Mass spectra were measured on an LKB-9000 gas chromatograph–mass spectrometer with a source temperature of 250°C and an electron beam voltage of 70 eV. Oxygenated steryl esters were admitted to the mass spectrometer via the direct insertion probe; saponified samples were admitted via either the direct insertion probe or the gas chromatograph inlet. In all cases, a 1% OV-17 column was used and the carrier gas was helium, at a flow rate of 20–40 ml. The temperature of the column typically was programmed up from 80°C at 5°C/min.

Radioactive compounds

[4-^{14}C]Cholesterol (53.4 mCi/mmole, New England Nuclear) was chromatographed on a column of 10 g of aluminum oxide, grade I (Woelm, Eschwege, Germany) prior to use. [4-^{14}C]Cholesteryl oleate (sp act 7.5 $\times$ 10^6 dpm/μmole) was prepared from [4-^{14}C]cholesterol and oleoylchloride (17).

7α-[7β-^{3}H]Hydroxycholesteryl oleate (sp act 2.4 $\times$ 10^7 dpm/μmole) and 7β-[7α-^{3}H]hydroxycholesteryl oleate (sp act 9 $\times$ 10^7 dpm/μmole) were prepared by reduction of 7-ketocholesteryl oleate with $NaBH_4$ (New England Nuclear). The labeled compounds were isolated from the isomeric mixture (70% 7β-[7α-^{3}H]hydroxycholesteryl oleate, 30% 7α-[7β-^{3}H]hydroxycholesteryl oleate) by column chromatography on silica gel and subsequent preparative TLC (petroleum ether–ether–acetic acid 90:10:1). In this solvent system, 7α-hydroxycholesteryl oleate (R_f 0.13) and 7β-hydroxycholesteryl oleate (R_f 0.15) separated from each other and cholesterol (R_f 0.1).

The purified hydroxycholesteryl esters ran as single spots in several thin-layer systems, viz., benzene–ethyl acetate 2:1 (v/v), petroleum ether–diethyl ether–acetic acid 90:10:1 (v/v), being detected by the blue color given when sprayed with 50% sulfuric acid in water or phosphotungstic acid (**Fig. 1**). Mass spectroscopy disclosed a molecular ion at *m/e* 666, which corresponds to the calculated molecular weight.

7-Ketocholesteryl [9,10-^{3}H]oleate (sp act 4 $\times$ 10^7 dpm/μmole) and 5,6α-epoxycholesteryl [9,10-^{3}H]oleate (sp act 4.4 $\times$ 10^7 dpm/μmole) were prepared from the corresponding sterols by acylation with [9,10-^{3}H]oleic acid (Radiochemical Centre, Amersham, England) (12). The compounds were purified by preparative TLC (petroleum ether–ether–acetic acid 90:10:1) and gave a single radioactive peak when chromatograms were examined in a radiochromatogram scanner, Packard model 7201. Structural identity was further confirmed by mass spectrometry (molecular ions of 7-ketocholesteryl oleate and 5,6α- and 5,6β-epoxycholesteryl oleate at *m/e* 664 and *m/e* 666, respectively). Radioactivity was assayed in a Packard Tri-Carb scintillation counter, model 3375. Appropriate corrections were made for quenching, crossover, and background.

RESULTS

When the neutral lipid fractions from livers of the patients with Wolman's disease were examined by analytical TLC (petroleum ether–ether–acetic acid 90:10:1), 10 distinct spots were detected on H_2SO_4-charred chromato-

plates (**Fig. 2**). Cholesterol, free fatty acids, di- and triglycerides, and cholesteryl esters were tentatively identified by position (R_f value), color reaction, and cochromatography with reference compounds. Confirmation of structural identity was obtained by GLC or MS, or both (see below).

After identification of these compounds, there remained five prominent spots on the TLC plates. These were designated as fractions 1–5, as indicated in Fig. 2. These unknown lipids were isolated by column chromatography. Fractions 1 and 2 eluted from a silicic acid column with 15%, fraction 3 with 10%, and fractions 4 and 5 with 5% benzene in hexane, respectively. Ultimate purification to a single TLC spot was obtained by repeated preparative TLC in petroleum ether–ether–acetic acid 90:10:1.

After the H_2SO_4-charred TLC plates were heated for 30 sec at 100°C, fractions 1 and 2 gave a characteristic blue reaction. Further heating caused these spots to turn black. In addition, both fractions 1 and 2 gave a strong positive Lifschütz color reaction (21). When the TLC plates were sprayed with a saturated solution of dinitrophenylhydrazine in concentrated sulfuric acid, fraction 3 turned yellow, indicative of hydrazone formation. Alkaline hydrolysis of fractions 1–5 resulted in the formation of both more polar lipids and fatty acids, indicating that each of these unknown compounds was a fatty acid ester (**Fig. 3**). The more polar lipids gave the same color reactions as their parent fractions prior to saponification. Purification of the lipids obtained after alkaline hydrolysis was achieved by preparative TLC using the solvent system hexane–ether 70:30 or ether–water 100:0.5. The fatty acid methyl esters obtained from fractions 1–5 by repeated hexane extraction of the acidified alkaline hydrolysis mixtures and subsequent diazomethane treatment gave almost identical patterns. Oleic acid was the most abundant fatty acid present (**Table 2**). These fatty acid patterns were also indistinguishable from those of cholesteryl esters in the same tissue (Table 2). Hydroxy fatty acid methyl esters could not be detected.

$NaBH_4$ reduction of fraction 3 (Fig. 2) produced a mixture of compounds with chromatographic properties identical with those of fractions 1 and 2. Treatment of fractions 1, 2, 4, and 5 with $NaBH_4$, however, did not change their chromatographic behavior. The unknown compounds and some of their derivatives were further analyzed by MS and NMR spectroscopy.

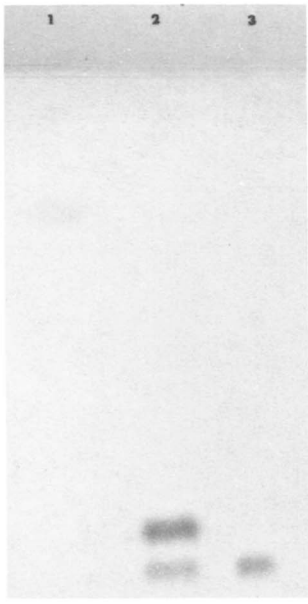

Fig. 3. Thin-layer chromatogram of native fraction 1 (lane 1) and fraction 1 after alkaline hydrolysis (lane 3). Lane 2, mixture of 7α-hydroxycholesterol (lower spot) and 7β-hydroxycholesterol (upper spot). Solvent system, ethyl ether–water 100:0.5.

Mass spectroscopic analysis

Mass spectral examination of the fractions eluted from the thin-layer spots was conducted by evaporating the samples from the direct insertion probe at the appropriate probe temperature. Because of the high mass of several of the compounds, the mass marker was calibrated with perfluorokerosene prior to use.

Fig. 4 shows the spectrum of fractions 1. Intense ions are observed at m/e 367 and 384, but the intensity of m/e 382 and 383 suggest that m/e 384 is not a molecular ion. Furthermore, the substance did not provide ions until the temperature of the probe was raised to $\sim$ 250°C, suggesting a much higher molecular weight; at higher gain, a se-

TABLE 2. Fatty acid composition of cholesteryl esters and oxygenated cholesteryl esters isolated from liver (patient D.D.)

	16:0[a]	16:1	1:80	18:1	18:2	Other
			% of total fatty acids			
Cholesteryl esters	21.0	14.2	4.4	29.8	25.1	5.5
7α-Hydroxycholesteryl esters	19.6	15.0	5.0	30.9	24.2	5.3
7β-Hydroxycholesteryl esters	22.0	13.8	3.9	33.9	21.9	4.5
7-Ketocholesteryl esters	23.1	12.8	5.6	30.1	24.4	4.0
5,6α-Epoxycholesteryl esters	21.1	14.0	6.1	34.3	22.0	2.5
5,6β-Epoxycholesteryl esters	18.7	13.9	5.3	29.0	25.0	8.1

[a] Number of carbon atoms:number of double bonds.

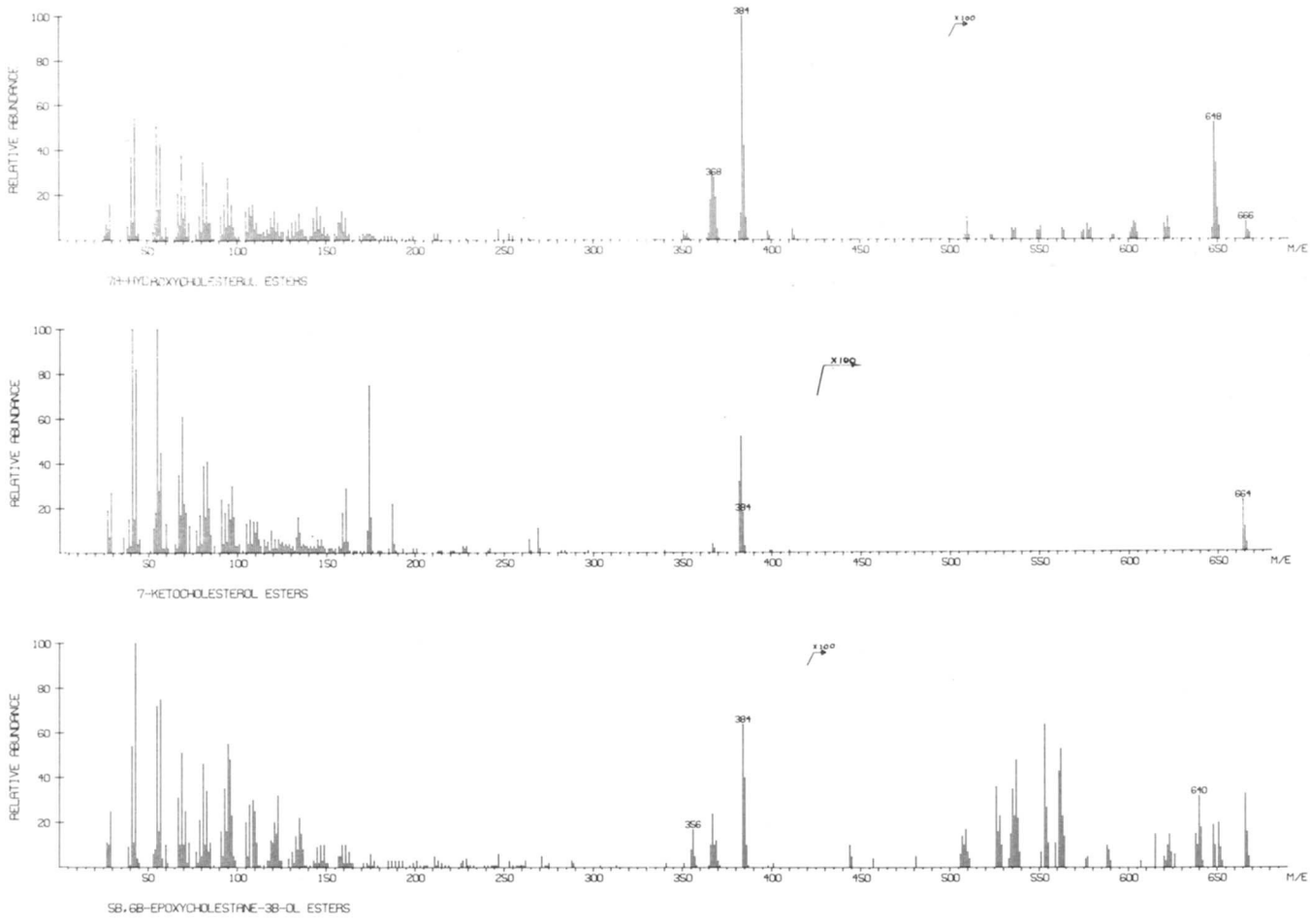

Fig. 4. Mass spectra of fraction 1 (*top*), fraction 3 (*middle*), and fraction 5 (*bottom*) (see Methods and Results).

ries of less intense ion clusters every 14 atomic mass units (amu) could be observed extending to m/e 648, suggesting a hydrocarbon chain. The ion at m/e 384 suggested the presence of a modified cholesterol (mol wt 386) nucleus. The gap from m/e 648 to m/e 384 corresponds to oleic acid minus water (264 amu). The fraction would thus seem to be a hydroxylated cholesteryl oleate of mass 666 that loses water (m/e 648) or oleic acid (m/e 384), or oleic acid plus water (m/e 367) (**Fig. 5**).

Fraction 2 exhibited a very similar spectrum, although in this case a very weak (<0.01%) molecular ion was also observed at m/e 666. Another weak ion at m/e 622 is considered to be the molecular ion of the lower homolog

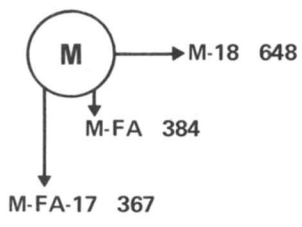

Fig. 5. Fragmentation patterns of 7-hydroxycholesterol esters. M, mass of parent compound; FA, fatty acid.

(palmitic acid). Undoubtedly, other homologs are actually present in both cases (see GLC analysis), but the ions in this region are so weak as to make intensity measurements unreliable.

Under the TLC conditions employed, it is unlikely that fractions 1 and 2 would have been resolved on the basis of carbon chain length only, and it seemed more reasonable to suppose that they were isomers.

Proof that a hydroxyl group was indeed present in both materials was obtained by preparing their trimethylsilyl ethers. Mass spectra of both were nearly identical. Weak but distinct molecular ions are observed at m/e 738 for the oleate component and m/e 710 and 712 for the palmitate and palmitoleate homologs. Loss of the acid moieties again gives an intense ion at m/e 456, which is the silylated counterpart of the ion seen at m/e 384 in the original compounds.

After saponification with alkali, each of the fractions again gave nearly identical mass spectra; that of the first is shown in **Fig. 6.** A weak molecular ion is observed at m/e 402, with an intense peak for the loss of water at m/e 384. Proof that the first peak was not an impurity was afforded by the intense metastable ion at m/e 367.5 for the

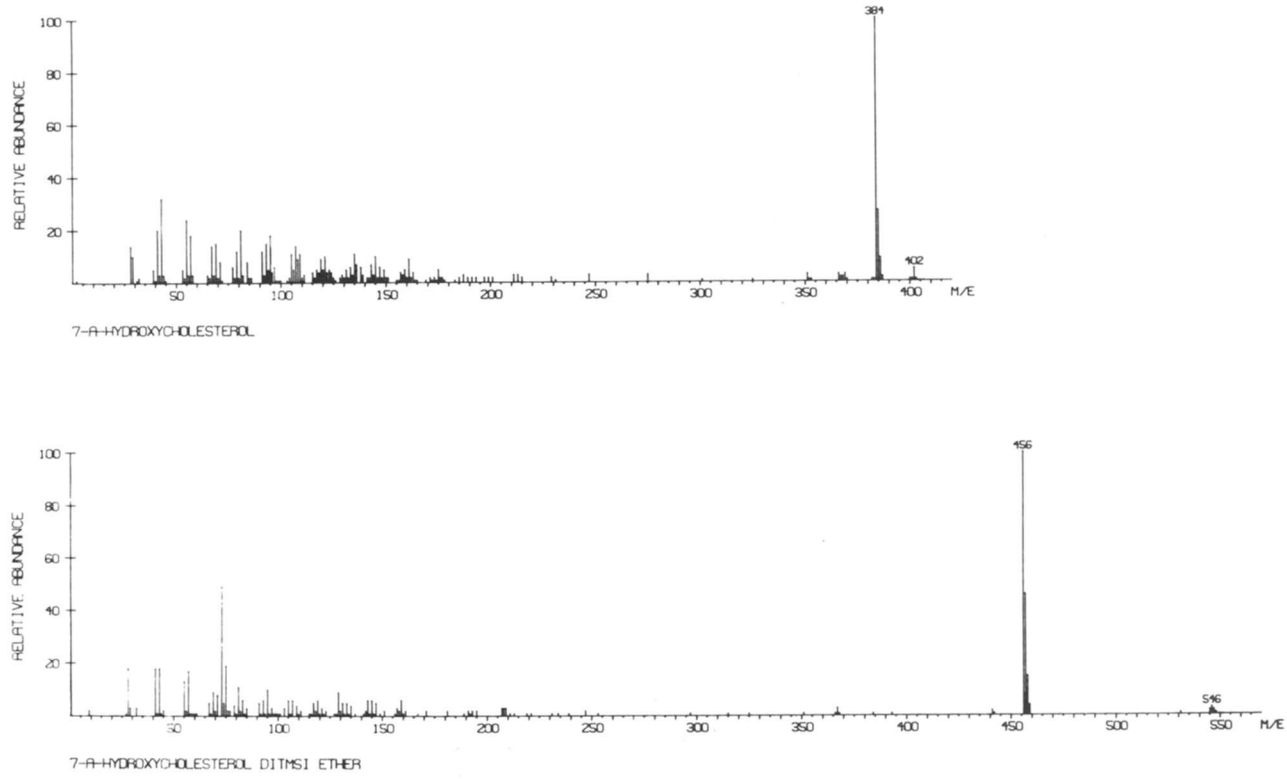

Fig. 6. Mass spectra of fraction 1 after alkaline hydrolysis (*top*), and TMSi ethers of the alkaline hydrolysis product (*bottom*):

transition 402 → 384 (calcd 367.5). This compound also formed a di-TMSi derivative showing an easily observable molecular ion at m/e 546 and a base peak at m/e 456 (M − TMSiOH) (Fig. 6).

Thus, the structure of a hydroxylated cholesteryl ester was confirmed, but the location of the substituents on the ring remained to be established. Catalytic hydrogenation of the hydrolysis product of the second fraction and silylation of the product fortuitously provided an answer. The molecular ion at m/e 460 and loss of TMSiOH (m/e 370) prove that a hydroxyl group has been lost in the reduction in addition to the expected saturation of the double bond. On comparing the spectrum with those present in the NIH computer search system, close fits were obtained with 5α- and 5β-cholestan-3α- and -3β-ols. Comparison of the retention times and mass spectra with authentic 5α-cholestan-3β-ol trimethylsilyl ether established their identity.

Loss of a hydroxyl group in this manner is characteristic of allylic alcohols in general and 7-hydroxy-5-cholestenes in particular (22), strongly suggesting that the hydrolysis products of fractions 1 and 2 were 5-cholestene-3β-, 7α-, or β-diols. This was confirmed by NMR analysis (see below), which also allowed assignment of the α and β configuration. Comparison with authentic samples by GLC (see below) and GLC–MS confirmed that fraction 1 corresponded to the 7α isomer and fraction 2 to the 7β isomer.

Because fraction 3 was reduced by $NaBH_4$ to fractions 1 and 2, it appeared to be the corresponding ketone mixture. This was further established by its mass spectrum, which showed a base peak at m/e 382 analogous to the ion at m/e 384 in the corresponding alcohols due to loss of the fatty acid moieties (Fig. 4). Weak molecular ions (<1%) were observed at m/e 610, 638, and 664 for the C_{14} and C_{16} saturated and C_{18} unsaturated esters, respectively. No ions were observed for loss of water in this case. Proof that this fraction contained keto esters was given by ready formation of the methoxime derivative in pyridine. The base peak shifted from m/e 382 to m/e 411 for the M − RCOOH fragment ion, corresponding to the conversion of C=O → C=NOCH$_3$. Fragment ions at m/e 380 and 381 for loss of OCH_3 and OCH_2 were also seen. A very weak (<1%) molecular ion was observed at m/e 693 for the C_{18} unsaturated homolog.

Hydrolysis of the keto ester fraction supplied the keto alcohol, which showed a molecular ion at m/e 400 as the base peak, along with loss of water (15%). This compound was found to be identical with 7-ketocholesterol in all respects, establishing that the original ester group was at the 3-position in both the original ketone and the corresponding 7-alcohols. NMR data confirmed this assignment (see below).

Finally, fractions 4 and 5 showed nearly identical mass spectra (Fig. 4). A weak molecular ion was observed at m/e 666 and a dehydration ion at m/e 648. (Peaks were

TABLE 3. NMR chemical shifts of cholesteryl esters and derivatives[a]

	Carbon Number, Proton Position							
	3	6	7	18	19	21	26, 27	Acyl
Cholesteryl esters	4.60	~5.30		0.68	1.02	0.92	0.87	5.30, t
7α-Hydroxycholesteryl esters	4.68, br	5.63, d J = 6 Hz	3.82	0.686	1.013	0.92	0.87	5.34, t
7β-Hydroxycholesteryl esters	4.60	5.30, m	3.84	0.695	1.06	0.91	0.87	5.3, m
7-Ketocholesteryl esters	4.70	5.70, s, br		0.685	1.205	0.92	0.86	5.35, t
5,6α-Epoxycholesteryl esters	4.95	2.85, d J = 4 Hz	3	0.71	1.08	0.93	0.87	5.35, t
5,6β-Epoxycholesteryl esters	4.80	3.06 J = 3 Hz		0.65	1.02	0.89	0.87	5.34, t

Abbreviations: J, coupling constant; s, singlet; d, doublet; t, triplet; m, multiplet; br, broad.
[a] Chemical shifts are given in ppm downfield from internal tetramethylsilene.

also observed for loss of the acid residue at m/e 384 and 368.) This suggested that the substance was related to the original fraction 1, but, unlike fraction 1, it did not form a trimethylsilyl ether and could not be considered an alcohol. Although the ions at m/e 384 and 385 indicated the presence of a second oxygen, a keto function was also ruled out because it failed to undergo reduction with sodium borohydride. An ether linkage was therefore indicated, and hydrolysis of the ester group of fraction 5 with alkali provided a compound that showed a molecular ion at m/e 402 and loss of water (m/e 384). As expected, a trimethylsilyl ether formed easily, showing a molecular ion at m/e 474 (100%) and loss of TMSiOH at m/e 384 (85%).

The presence of 7-ketocholesterol suggested that these ethers were epoxides related to this substance. Sufficient sample was collected to prove the presence and configuration of the epoxy compound by NMR (see below), and fractions were then compared with authentic compounds, proving that fractions 4 and 5 were esters of cholestane-5α,6α-epoxy-3β-ol and cholestane-5β,6β-epoxy-3β-ol, respectively.

Nuclear magnetic resonance spectroscopy

The position and stereochemistry of the substituents on the sterol ring structure were recognized by the chemical shifts and coupling patterns of the associated protons and by the chemical shifts of the angular methyl groups (**Table 3**). The presence of a 7 substituent in 7α-hydroxycholesteryl esters is revealed by the appearance of the C-6 proton and a doublet; there is very little effect upon the angular methyl groups, and the observed chemical shifts are otherwise identical with those obtained with cholesteryl ester. In the 7β epimer, the anticipated effect of a vicinal equatorial group shifts the C-6 proton upfield to 5.3; there it coincides with the olefinic absorption of the fatty acid and cannot be seen distinctly. The anticipated downfield shift of both methyl groups is observed. In the absence of any vicinal protons with which to couple, the C-6 proton of the 7-keto derivative appears as a singlet. The striking downfield shift of the C-19 protons and absence of shift of C-18 protons correspond to previously observed effects (23).

In the spectra of the two epoxides, the presence of doublets near 3 ppm is attributable to a proton on a carbon bearing an oxygen, the proton resonance having been shifted upfield from the more customary carbinol position at 3.8 ppm; the presence of the three-membered ring of the epoxide explains the anomalous high field position. The observed chemical shifts and those of the angular methyl groups correspond well to the values reported for the corresponding acetates (24). The spectrum of cholesteryl esters isolated from autopsy tissues of patients with Wolman's disease was essentially indistinguishable from that of the reference compounds.

Gas–liquid chromatography

Further confirmation of the stereochemical identity of 7α- and 7β-hydroxycholesteryl esters was achieved by GLC of the alkaline hydrolysis products, 7α- and 7β-hydroxycholesterol, on 3% OV-210 as the liquid partition phase. The quasi-axial 3β,7α-diol was eluted ahead of the quasi-equatorial 3β,7β-diol, and both cochromatographed with reference compounds. Their retention times relative to cholesterol were 2.16 and 2.34, respectively. The 5,6α-

TABLE 4. Neutral lipid composition of liver in Wolman's disease

	Patient	
	D.D.	A.J.
	mg/100 mg dry tissue	
Cholesteryl esters	11.4	9.8
Cholesterol	3.0	2.1
Triglycerides	13.0	12.5
Free fatty acids	0.6	0.4
7α-Hydroxycholesteryl esters	0.8	0.7
7β-Hydroxycholesteryl esters	0.5	0.3
7-Ketocholesteryl esters	0.2	0.3
5,6α-Epoxycholesteryl esters	0.1	0.1
5,6β-Epoxycholesteryl esters	0.2	0.3

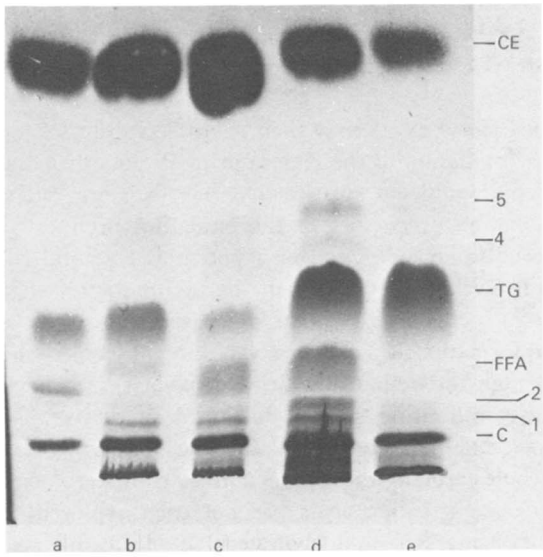

Fig. 7. Thin-layer chromatogram of neutral lipids from adrenal, liver, and spleen. Solvent system, petroleum ether–ether–acetic acid 90:10:1. *a,* Adrenal (control tissue); *b,* adrenal (patient A.J., Wolman's disease); *c,* adrenal (patient D.D., Wolman's disease); *d,* liver (patient D.D., Wolman's disease); *e,* spleen (patient A.J., Wolman's disease).

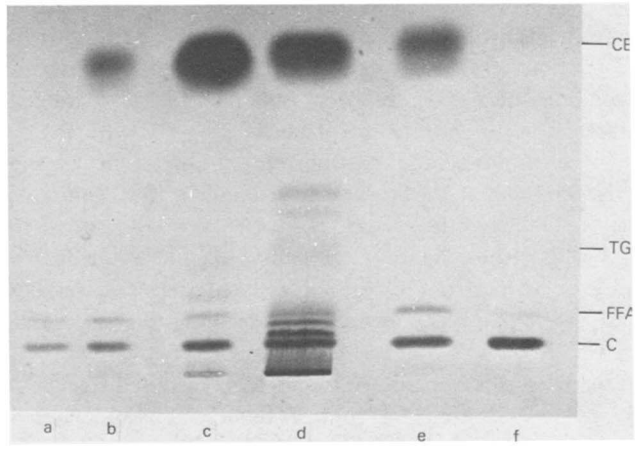

Fig. 8. Thin-layer chromatogram of tissue lipids. Solvent system, petroleum ether–ether–acetic acid 90:10:1. *a,* Tonsil (patient H.M., Tangier heterozygote); *b,* tonsil (patient E.L., Tangier homozygote); *c,* liver (patient L.Mc., cholesteryl ester storage disease); *d,* liver (patient D.D., Wolman's disease); *e,* spleen (patient C.N., Tangier disease); *f,* brain (patient L.Mc., cholesteryl ester storage disease).

and 5,6β-epoxycholesterol epimers could not be satisfactorily resolved on GLC.

Tissue concentrations of the oxygenated sterols

To determine the tissue concentrations of the oxygenated steryl esters, reference compounds of high specific activity were synthesized by esterifying the sterols with [9,10-^{3}H]oleic acid (see Materials and Methods). These were added separately to liver homogenates. The latter were lyophilized and extracted with organic solvent. The compounds were collected in ultimate purity from TLC and weighed individually, and their concentrations were determined by corrections for loss of radioactivity (**Table 4**).

The presence of the oxygenated steryl esters in spleen and adrenals of the same patients with Wolman's disease was ascertained only qualitatively by TLC (**Fig. 7**). The absolute tissue concentration of these compounds was greatest in the liver; given the very high concentrations of cholesteryl esters in the adrenals, it was apparent that the concentration of oxygenated steryl esters in the several tissues was not simply proportional to the cholesteryl ester concentration.

All of the control tissues were extracted and the neutral lipid fractions obtained from each in a manner identical with that used for tissues of patients with Wolman's disease. In none of these control tissues was any trace of oxygenated steryl esters detected by TLC. Chromatography of representatives among these control tissues is illustrated in Fig. 7 and **Fig. 8.** The cholesteryl ester concentrations in the tonsils of the patients with Tangier disease (18–25 mg/g wet wt [10]) and in the liver of the patient with cholesteryl ester storage disease (187 mg/g) were comparable to or exceeded those in the liver of the Wolman's disease patients (Table 1).

DISCUSSION

Oxidation products of cholesterol have been isolated from a number of natural sources (25–28). There is very little information available, however, concerning the occurrence in biological material of compounds that are either oxidation products of cholesteryl esters or result from esterification of oxygenated sterols. Small amounts of an ester of cholest-5-ene-3β,7-diol have been isolated from rat liver (29) and obtained after the incubation of cholest-5-ene-3β,7α-diol with the soluble fraction of rat liver homogenate (30).

It is noteworthy that published chromatograms (31) of lipid extracts from liver and spleen of two other patients with Wolman's disease appear to us to contain compounds similar to those identified in the present communication. Despite this additional suggestion that a perhaps unique accumulation of oxygenated steryl esters is a universal phenomenon in tissues from patients with Wolman's disease, it is paramount to consider the possibility that this may be solely the result of autoxidation occurring in the storage of tissue postmortem.

It is well known that cholesterol is subject to autoxidation in the colloidally dispersed state. Bergström and Wintersteiner (32), using an aerated aqueous dispersion of cholesterol, identified the major oxidation products as 7-ketocholesterol, 7β-hydroxycholesterol, 7α-hydroxycholesterol, and 3,5-cholestadiene-7-one. In addition to the

above-mentioned compounds, cholestane-3β,5α,6β-triol (33), cholesterol-5α,6α-epoxide (34), and cholesterol-5β,6β-epoxide (35) have been isolated from air-oxidized cholesterol as minor reaction products.

We cannot exclude postmortem autoxidation as the source of some of the steryl esters found in the Wolman's tissues. On the other hand, there are several reasons to suspect that the oxidation probably took place during life and that at least one of the oxygenated sterols arises because of the pathologic processes underlying this particular disease.

One reason is our failure to detect oxygenated steryl esters in several kinds of control tissues. These included some that are usually rich in cholesteryl esters, such as adrenal, brain, and liver from controls and from a patient with cholesteryl ester storage disease and tonsils from patients with Tangier disease. Crucial differences in handling these organs subsequent to their removal cannot be excluded, but many were retained in the frozen state for periods longer than the Wolman's tissues. Once thawed in the laboratory for analysis, care was taken to handle them in the same way. We have considered that the physical state of the cholesteryl esters stored in Wolman's disease might particularly favor autoxidation. Crystals of esters have been reported in light and electron micrographs of the Kupffer cells and adrenal histiocytes in this disease (36). Crystals of cholesteryl esters, however, are also demonstrable in tonsils and other tissues in Tangier disease (37).

A second argument against simple autoxidation is provided by both the close similarity of the fatty acid compositions of the cholesteryl esters and the oxygenated steryl esters in Wolman's disease and the absence of any detectable unesterified oxidation products of cholesterol. It has been observed that the saturated and monoenoic fatty acyl esters of cholesterol are almost completely resistant to autoxidation of the sterol moiety (38, 39). Like unesterified cholesterol, however, the dienoic and other polyunsaturated fatty acyl esters of cholesterol are more vulnerable to autoxidation in aqueous colloidal suspension (39, 40). Thus, autoxidation should have been expected to have produced both oxygenated unesterified sterols and a disparity between the fatty acyl compositions of the cholesteryl and oxygenated steryl esters in Wolman's disease tissues.

The alternative to autoxidation is enzymatic oxidation. Recent studies from several laboratories have suggested that oxygenated sterols such as 7-ketocholesterol, 7α- and 7β-hydroxycholesterol, and cholestane-3β,5α,6β-triol can be formed by microsomal NADPH-dependent enzymatic lipid peroxidation, at least in liver (41, 42). The steryl esters, too, may be substrates for enzymatic oxidation. Cholesteryl palmitate has been shown to be metabolized to cholesterol-5α,6α-epoxide and cholestane-3β,5α,6β-triol by rat brain subcellular fractions (43). This conversion could not be demonstrated for cholesterol, suggesting that cholesteryl ester itself reached the site of microsomal oxidation.

Thus, steryl esters may themselves be substrates for enzymatic oxidation of the ring, and in Wolman's disease it seems reasonable to suspect that one or more esters are probably the precursors of the oxidation products. This brings us to consideration of a potentially pivotal role of 7α-hydroxycholesteryl ester in the accumulation of lipids that seems peculiar to Wolman's disease.

The formation of 7α-hydroxycholesterol from cholesterol, through the action of a 7α-hydroxylase, is believed to be the rate-limiting step in bile acid synthesis (44–46). The metabolic significance of fatty acid esters of 7α-hydroxycholesterol in the conversion of cholesterol or cholesteryl esters to bile acids has not been explored. Boyd (29) has suggested that cholesteryl linoleate may be the precursor in 7α-hydroxycholesterol formation. Swell and Law (47, 48) observed a more rapid conversion to bile acids of linoleic and arachidonic esters for [4-^{14}C]cholesterol than of palmitic and oleic esters.

It is conceivable that Wolman's disease involves a block in the conversion to bile acids of 7α-hydroxycholesterol itself and that this compound is completely esterified as it accumulates. We consider to be more probable, however, the alternative possibility that cholesteryl ester may be hydroxylated at position 7 and that hydrolysis of the fatty acid from the 7α-hydroxycholesteryl ester may then be the next step required for further movement of this compound along the route of bile acid formation. Were hydrolysis of the ester blocked, the oxygenated steryl ester should accumulate. It might then become a precursor of other oxygenated steryl esters through either autoxidation or enzymatic oxidation.

We examined the validity of this second hypothesis by performing two experiments not reported heretofore.[1] It was first determined in vitro that rat liver cytoplasm contains enzymatic activity necessary for hydrolysis of 7α-hydroxycholesteryl oleate. We then demonstrated in a dog with a complete bile fistula that 7α-[7β-^{3}H]hydroxycholesteryl oleate is efficiently converted to cholic acid and, to a lesser extent, chenodeoxycholic acid. These data, however, do not permit conclusions concerning the physiological importance of 7α-hydroxycholesteryl ester as a precursor for bile acids. In fact, demonstration that isotopically labeled cholesterol is converted to 7α-hydroxycholesteryl ester is required to establish this compound as an intermediate in bile acid biosynthesis. Nevertheless, these considerations make it imperative that the synthesis of bile acids be examined in Wolman's disease. The steatorrhea and distention common in these patients has not been explained.

[1] Assmann, G., and D. S. Fredrickson. Unpublished data.

To be acceptable, the above theory still requires further explanations. For example, there is the absence of oxygenated steryl esters in the liver of one patient with cholesteryl ester storage disease, a disorder in which cholesteryl ester hydrolase activity is also deficient (3). These patients survive infancy and do not have malabsorption, although their bile acid patterns have been described as abnormal (49). A more difficult problem is posed by the presence of 7α-hydroxycholesteryl esters and the other oxygenated steryl esters in nonhepatic tissues in Wolman's disease. It may be that 7α-hydroxycholesteryl ester is also an intermediate in biochemical reactions in the adrenal, but its presence in the spleen would seem to require transport of oxygenated sterols and their esters in the plasma. This has not been examined.

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