Characterization of Some Unidentified Components in Aorta Lipids

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CHARACTERIZATION OF SOME UNIDENTIFIED COMPONENTS IN AORTA LIPIDS

by

Max E. Royer

A Thesis
Submitted to the
Faculty of the School of Graduate Studies in partial fulfillment of the
Degree of Master of Arts

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November 1969
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CHARACTERIZATION OF SOME UNIDENTIFIED COMPONENTS IN AORTA LIPIDS.

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INTRODUCTION

Atherosclerosis, a disease of modern man, is typified by the deposition of lipids on inner layers of arteries. Although aortic atherosclerosis is less frequently a cause of death than coronary or cerebral atherosclerosis, the aorta is the largest and the most accessible artery and is generally used to predict the extent of atherosclerosis in other arteries.¹

Much attention has been given to analysis of the major lipids of aortic plaques; namely, cholesterol, cholesteryl esters, triglycerides, sphingomyelin, and glycerophospholipids. Because of the difficult separation problems, less success has been achieved in characterizing the minor lipid components. The gas chromatograph and the mass spectrometer are becoming valuable tools for characterizing and identifying these less abundant lipids. Methods of derivatizing complex lipids to make them more volatile and stable in the gas chromatograph have enabled analysis of small amounts of high molecular weight lipids.² A more complete picture can be obtained of lipids found in plaques as better tools and methods become available.

Foote and Coles³, in their isolation and identification of cerebrosides from human aorta, found several uncharacterized components by thin-layer chromatography (TLC) which migrated faster than cerebrosides. With the system 24:7:1 chloroform:methanol:water (C:M:W)⁴, cerebrosides migrated with an Rf of about 0.5 and other components had Rf's of 0.7-0.85. Further investigation of these faster-migrating materials revealed that some carbohydrate may have
been present in this lipid, using the anthrone test.\textsuperscript{5} The research objectives of the current project were to further characterize and/or identify these lipid components (or unknown substances) which are apparent, ahead of cerebrosides, on a TLC plate when aorta lipids are purified by the method of Foote and Coles.\textsuperscript{3} The problem, therefore, became to determine which lipids: (1) would elute from a Florisil column with the neutral glycolipid fraction; (2) would migrate beyond cerebrosides using this TLC system; (3) could survive mild alkaline methanolysis; and (4) might contain a carbohydrate-like moiety.

A preliminary approach to this identification was to hypothesize what types of lipids would have the properties of the components observed in this TLC zone, then to test known compounds to find out where they migrate in this system. The material must be able to survive alkaline methanolysis and be less polar than cerebrosides. The substance must not be eluted from Florisil with 8:2 hexane ether (H:E), but should be easily eluted with the 3:1 chloroform:methanol (C:M) fraction which contains cerebrosides.

A survey of the literature revealed three Florisil chromatography systems concerning the separation of substances with migration rates similar to cerebrosides. Rouser\textsuperscript{6} reported the following elution sequence of brain lipids from dry Florisil: chloroform eluted cholesterol, 19:1 C:M eluted ceramide, 2:1 C:M eluted cerebroside and sulfatide, and methanol eluted phosphatidyl ethanolamine and ganglioside. Lecithin, sphingomyelin, lysolecithin, phosphatidyl
serine, and inositol phosphatide were eluted only after 7% water was added to the 2:1 C:M. With the exception of the last solvent system, all of the above solvents contained 5% by volume 2,2-dimethoxypropane to insure dryness. Creech\(^7\) reported separation of neutral lipids using Florisil activated at 600°C and containing 7% added water. The order of elution using hexane-ether and ether-methanol solvents was cholesteryl palmitate, tripalmitin, cholesterol, dipalmitin, and monopalmitin, followed by fatty acids. Fatty acids were not eluted quantitatively unless acetic acid was added. Coles\(^8\) reported that triglycerides and cholesterol were readily eluted from Florisil with 8:2 H:E free from any detectable cerebrosides. However, the 3:1 C:M fraction containing the cerebrosides also contained some cholesterol and phospholipid. After methanolysis, the phospholipid was converted to glycerol phosphate and choline. These were removed in the subsequent Folch wash. The methyl esters and the remaining cholesterol were almost totally removed by the 8:2 H:E in the subsequent Florisil column. Young\(^9\) separated glycolipids by TLC using 65:25:4 C:M:W, which is similar to the 24:7:1 C:M:W used by Foote and Coles.\(^3\) Ceramide, in his system, moved just behind the solvent front and well ahead of cerebroside. Free fatty acid migrated slightly behind cerebrosides.

Considering what compounds could be present in the TLC zone under investigation, phospholipids are eliminated as candidates by chromatography and methanolysis. Cholesterol, other sterols, or steroids could possibly be present after methanolysis and Florisil
chromatography. Ceramides or ceramide-like compounds would survive alkaline methanolysis and elute after the 8:2 H:E fraction, eluting after cholesterol. Free fatty acids might possibly be present after the methanolysis. These could elute in low yield from Florisil. Thus, some known lipids having the properties of these unidentified components are cholesterol (sterols) and ceramides. Other unknown substances may also be present in this lipid preparation.
MATERIALS AND METHODS

Materials

Chloroform and methanol used for extractions were redistilled, then mixed and stored in dark-brown bottles with Teflon or aluminum cap liners. All glassware and equipment used for extraction was rinsed just prior to use with 2:1 C:M to avoid possible contamination. The hexane was distilled in glass (Burdick and Jackson, Muskegon, Michigan). Other solvents were reagent-grade.

The Florisil (magnesium silicate) used for column chromatography was 60-100 mesh (The Floridin Co., Berkeley Springs, W. Va.). This was heated to 600°C for one hour, cooled in a desiccator, and 8% water by weight was added. After standing overnight, the Florisil was ready for use and was kept in this form for up to one month.

Pre-coated TLC plates were silica gel (Merck) containing F-254 phosphor (Brinkmann Instrument, Inc., Westbury, New York).

Reagents used to visualize zones on TLC plates were the following:

1. Bromthymol Blue spray -- 0.04% in 0.01 N NaOH
2. Vanillin phosphoric acid spray -- 1 gm vanillin, 100 ml 95% ethanol, and 50 ml of 85% phosphoric acid. Colored spots are visualized over about a ten-minute period while heating at 110°C.
3. Iodine vapor (ultraviolet optional).

Known compounds used for standards on TLC were lecithin (egg), cholesterol, diolein, oleic acid, cholesteryl palmitate, sphingo-
myelin (bovine), cerebrosides (bovine), and ceramides (mixed)
(Applied Science Laboratories, Inc., State College, Pa.). Batyl and
chimyl alcohols were from a crude preparation of ratfish oil.

Tissues

Human aortas were embalmed autopsy specimens obtained from
Borgess Hospital, Kalamazoo, Michigan. They were kept frozen until
ready for use. All aortas showed some degree of atherosclerosis in
stage II or stage III, ranging from small fatty lesions to large
plaques and loose calcified material with signs of thrombosis and
ulceration. Many of the plaques extended through the media, so that
the intima and media were not distinguished or separated. Material
from arterial aneurysms was not included in the lipid extract.
Since large amounts of lipid were needed for identification of minor
components, the fatty materials from several aortas were combined
for extraction of the lipids. Two preparations of aortas were in­
vestigated (A and B). Groups A and B contained the plaque materials
from four and two aortas, respectively. Gross inspection indicated
that the group B aortas were more atherosclerotic than the group A
aortas.

Isolation of Material to be Studied

Extraction of lipids

The lipids were extracted by the method of Folch using 2:1 C:M.
The lipid-containing material was ground and stirred with the C:M in
a large glass vessel for at least five minutes using a Polytron wet-
milling mixing device (Bronwill Scientific, Rochester, N. Y.). After filtering through a glass-frit filter, the Folch extraction was carried out using one-fifth volume of 0.1 N KCl. The two phases were allowed to separate for about one hour at 5°C; then the lower phase was collected, about one-fifth volume of absolute ethanol was added, and the samples were dried at 35°C on a Büchi Rotavapor (Rinco Instrument Co., Inc., Greenville, Ill.). Weighed samples were re-dissolved in absolute ethanol and redried in tared flasks. The lipids were stored at 5°C in a small volume of 2:1 C:M. Each aorta yielded from 2-15 gms of crude lipids.

Column chromatography - initial purification

Florisil chromatography was carried out essentially as described by Foote and Coles. A 100-ml burette fitted with a Teflon stopcock and a needle valve was found to be satisfactory as a column. To avoid possible contamination from stopcock grease, only Teflon stopcocks or new glassware with unlubricated stopcocks were used. The filter at the bottom of the column was glass wool covered with a layer of sand. Florisil was poured into the column which contained 8:2 H:E and packed by tapping the column sides. About an inch of sand was placed on top of the Florisil. This aided in sample application and in changing from the less dense H:E to the more dense C:M. The sample was applied to the column by dissolving the lipid in a minimum volume of 8:2 H:E and carefully layering the solution on the sand with a long pipette. The columns were developed at a rate of about 1 ml/min, depending on the column diameter.
Ten gms of Florisil were used per gm of crude lipid for the initial cleanup of the wanted lipid materials. The nonpolar lipids, mainly triglyceride, cholesteryl esters, and cholesterol, were eluted with 8:2 H:E (25 ml/gm of Florisil). The fraction containing predominantly cerebrosides and the uncharacterized materials under study was then eluted with 3:1 C:M (25 ml/gm of Florisil).

**Alkaline methanolyis**

After drying and weighing the 3:1 C:M eluate from the first column, a mild alkaline methanolyis was carried out to convert the fatty acid of any glycerophosphatide or triglyceride to methyl esters by transesterification. This mild reaction does not break the fatty acid-amide bond found in sphingolipids. One part of 0.21 N NaOH in methanol was mixed with two parts of chloroform. This was added to the lipid (10 ml per 30 mg) and stirred for one hour at room temperature. To this mixture was added one-fifth volume of 2% acetic acid. This neutralized the NaOH and made the Folch partition. After drying the lower layer, the lipids were ready for further purification by Florisil chromatography.

**Florisil chromatography**

After methanolyis, the methyl esters and some of the remaining nonpolar lipids were separated on a small Florisil column. Forty gms of Florisil were used per gram of lipid. Except for its smaller size, this column was developed in the same manner as the Florisil column used in the initial cleanup. In the remainder of this study,
the last 3:1 C:M fraction of lipids will be referred to as the lipids from the Florisil column.

**Separation of lipids by preparative TLC**

The lipids from the Florisil column were further purified using preparative TLC plates (a 2-mm layer of silicic acid) and a solvent system of 24:7:1 C:M:W. These plates were pretreated to remove possible contaminants by developing them to the top with 2:1 C:M, then drying, and reactivating in a 110°C oven. When visualized with the bromthymol blue spray, five major bands were visible. The zone containing the lipids under study had an $R_f$ of about 0.7 in this system. It was well separated from cerebrosides but poorly separated from the material streaked above it. In the remainder of this study, the cerebrosides band from 24:7:1 C:M:W will be referred to as zone 2 and the two major zones migrating well above cerebrosides as zones 4 and 5.

For some experiments, zone 4 was eluted and rechromatographed on a 0.5-mm preparative TLC plate, using a system of 95:5 C:M. This separated zone 4 into six bands. The bands of this latter TLC system were marked with a pencil and scraped from the plates using a spatula. These materials were packed into columns with glass-wool plugs in the bottom. The lipids were eluted directly into separatory funnels using 150 ml of 2:1 C:M. Thirty ml of 0.1 $N$ NH$_4$OH were then added and the resulting Folch partition removed the bromthymol blue salt from the lipids. The lower layer was dried on the rotary evaporator.
and the lipids were transferred to small glass-stoppered tubes with 2:1 C:M.

Gas-Liquid Chromatography (GLC)

General method

An F & M Model 402 gas chromatograph (Hewlett-Packard Co., Avondale, Pa.) equipped with dual flame ionization detectors was used for most of the gas chromatographic separations. To allow well-controlled programming at high temperatures, two 1200-watt heaters were installed in the column oven. The injection port and detector temperatures were kept at least 20°C above the column temperature. A 1.5-foot column containing 1% OV-1 on GasChrom Q (Applied Science Laboratories, Inc., State College, Pa.) was used for most of the high-temperature work. Carrier gas was helium. Peak areas were measured by planimetry.

Trimethylsilylation

A small amount of lipid solution was dried with nitrogen in a conical centrifuge tube. This was dissolved in about 0.1 ml of bis-trimethylsilyl trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA + 1% TMCS) (Regis Chemical Co., Chicago, Ill.). In cases where the compounds would not dissolve, a drop or two of dimethylformamide was added to dissolve them. Some solutions were gently warmed for ten to 30 minutes. Others were allowed to stand at room temperature for an hour or more. They could then be injected directly into the gas chromatograph. Silylated ceramides were eluted as
symmetrical peaks while the chromatograph was temperature-programmed from 250° to 350°C, or at other suitable rates and temperatures.

**GLC of fatty acids after acid methanolysis**

The materials from zone 4 and the cerebroside zone were subjected to acid methanolysis by the method of Vance and Sweeley. The methyl esters of fatty acids were recovered from the methanolic HCl by adding 150 ml of 2:1 C:M and 30 ml of 0.1 N NH₄OH. The methyl esters were extracted into the lower phase and dried on the rotary evaporator.

The methyl esters of fatty acids were identified by GLC on two columns by comparing the retention times with standards of the methyl esters of fatty acids 20:0, 20:1, 22:0, 22:1, 24:0, and 24:1 (K-107, Applied Science Laboratories, Inc.). The polar column used for this separation was 6% diethylene glycol succinate (DEGS) on Diatoport S (Hewlett-Packard Co.). A four-foot column containing 3% SE-30 on 80-100 mesh GasChrom Q (Applied Science Laboratories, Inc.) served as the nonpolar column.

**GLC-mass spectrometry**

A two-foot 1% OV-1 column was conditioned at 350°C for 24 hours in the F & M 402 gas chromatograph. It was then installed on an LKB-9000 gas chromatograph-mass spectrometer (LKB Instruments, Inc., Stockholm, Sweden) and again conditioned overnight at 280°C. The settings on the mass spectrometer were as described by Samuelsson, with the exception that most samples were ionized at 70 electron volts. The separator temperature was kept at 290°C for the high-
temperature work and at 265°C for the low-temperature work. If significant column bleed was present, background spectra were run. Early spectra were counted, beginning with the oxygen m/e 32 peak. Later, a mass marker was used. The more volatile components were separated at 220°-250°C on a six-foot column containing UC W-98 on 80-100 mesh GasChrom Q (Applied Science Laboratories, Inc.).

Sugar Analysis

Zone 4 and the zone migrating immediately above it (24:7:1 C:M:W) were eluted from a preparative TLC plate and were subjected to 16 hours of acid methanolysis at 75°C in 3 ml of 1.5 N methanolic HCl. After addition of 0.35 ml of water, the methanol phase was extracted four times with n-hexane. This removed the methyl esters. The methanol phase was then percolated over an Amberlite CG-4B column to remove the HCl, and the methanol phase was dried on the rotary evaporator.

If present, sugars were converted to their respective trimethylsilyl ethers by warming the samples gently with trimethylsilylimidazole (TRI-SIL “Z”, Pierce Chemical Co., Rockford, Ill.). A four-foot 3% SE-30 column was used to chromatograph the silylated sugars. Glucose, galactose, and mannitol were used as standards.
RESULTS AND DISCUSSION

Isolation Procedures

The 24:7:1 C:M:W system used by Foote and Coles\textsuperscript{3} and by Tao\textsuperscript{16} is an excellent system for separation of cerebrosides and the other lipids from the Florisil columns. However, this system lacks the resolution needed for separation of less polar components which move farther up the plate. As can be seen in Figure I, ceramides, oleic acid, and cholesterol are not distinguished by this system. It soon became apparent that the zone 4 material consisted of more than one component.

Two approaches were used for TLC separation of the components of interest from the Florisil columns. First, solvent systems less polar than the 24:7:1 C:M:W which would discriminate in the region of cholesterol and ceramide were tried. Second, zone 4 was recovered and rechromatographed in systems that hopefully would resolve zone 4.

Pursuing the first approach, the water in the 24:7:1 C:M:W system was replaced with \textit{NH}_4\textit{OH}. This held the fatty acid standard near the origin, but did not greatly affect the resolution or the \textit{R}_f's of the other components. Decreasing the amount of water (or \textit{NH}_4\textit{OH}) by a factor of two (24:7:0.5) decreased the \textit{R}_f's slightly, but did not change the relative positions of ceramide or cholesterol standards.

In an attempt to resolve ceramide and cholesterol, several other solvents and solvent systems with dielectric constants in the same
A and B are lipid preparations from the Florisil column. A-f2, A-f4, A-f5 are zones 2, 4, and 5, respectively, as recovered from preparative TLC. The solvent was 24:7:1 C:M:W. Staining was by iodine vapor and the photograph was taken under ultraviolet light. Note zone 4, used for most of the studies, had an $R_f$ of about 0.7, but does not appear as a single component.
range as 24:7:1 C:M:W were used to separate standards of cerebroside, ceramide, and cholesterol. Systems containing acetone instead of alcohol separated cholesterol from the upper ceramide component (Table I). Considerable streaking was apparent in these systems, and the resultant overlap would not have given pure components in a preparative TLC system. Substituting ethanol for methanol gave only slightly improved separation of the upper ceramide component and cholesterol.

Table I. Solvent Systems for Separation of the Lipids from the Florisil Columns - Rf Values of Components

<table>
<thead>
<tr>
<th>Solvent System*</th>
<th>Ceramides**</th>
<th>Cholesterol</th>
<th>Cerebrosides**</th>
<th>Oleic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.23 S</td>
<td>0.65 S</td>
<td>0.85</td>
<td>- 0.0 -</td>
</tr>
<tr>
<td>II</td>
<td>0.60 S</td>
<td>0.74</td>
<td>0.82</td>
<td>0.0 0.2 0.6</td>
</tr>
<tr>
<td>III</td>
<td>0.78</td>
<td>0.87</td>
<td>0.89</td>
<td>0.51 0.55 0.60</td>
</tr>
<tr>
<td>IV</td>
<td>0.78</td>
<td>0.86</td>
<td>0.89</td>
<td>0.33 - 0.43</td>
</tr>
<tr>
<td>V</td>
<td>0.38 S</td>
<td>0.55 S</td>
<td>0.81</td>
<td>- 0.0 -</td>
</tr>
</tbody>
</table>

S = Streaked
* = Solvent Systems:

I 10:10:0.5 chloroform:acetone:28% NH₄OH
II 10:10:0.5 chloroform:acetone:water
III 25:7:0.5 chloroform:methanol:water
IV 25:7:0.5 chloroform:ethanol:water
V 20:0:5 methyl isobutyl ketone:water

** = Two zones were apparent for ceramides and up to three zones for cerebrosides.

Using solvent system I (Table I), the lipids from the Florisil columns showed five zones which migrated between hydroxyceramide and cholesterol standards. No zone corresponded to either of these
components; however, one zone was apparent which matched the non-hydroxyceramide standard.

The lipids from the Florisil columns were also chromatographed in a TLC system designed to separate sterols. In this system, consisting of 8:2 ethyl acetate:benzene, the cholesterol standard migrated with an Rf of about 0.6. The cerebroside remained at the origin. Most of the ceramide also remained at the origin; however, there was some streaking. In this system, the lipids from the Florisil columns showed material at the origin with some streaking. In addition, there were three zones with Rf's of 0.33, 0.43, and 0.54, and a large streak extending from Rf 0.7 to near the solvent front. No zone corresponded to the cholesterol standard.

Using the second approach to purify these components by TLC, a sizable quantity of zone 4 lipids was needed. Using 24:7:1 C:M:W in preparative TLC, the lipids from the Florisil columns separated into five zones. These zones were:

1. A zone which migrated behind OH-cerebroside standard -- Rf \approx 0.20.
2. A zone which migrated with standard galactosyl cerebroside, tentatively identified as a glucosyl cerebroside -- Rf \approx 0.40.
3. A zone containing only a trace of lipid, migrating ahead of cerebrosides -- Rf \approx 0.47-0.58.
4. A large zone migrating in the region of ceramide and cholesterol standards. This zone is called zone 4 and contains the materials of interest -- Rf \approx 0.58-0.75.
Another large zone not clearly separated from zone 4. It streaked from zone 4 to near the solvent front — Rf > 0.75. These zones on preparative (2-mm thick) TLC plates were similar to the zones in Figure I, but not as well resolved in the region of zones 4 and 5. Similar zones were found by Foote and Coles in their aorta lipid preparations.³

Karlsson¹⁸ successfully separated ceramides derived from sphingomyelins of bovine stomach rennet, using a system of 95:5 chloroform: methanol. Ceramides from hydrolysis of sphingomyelins separated into two pairs of bands. The fastest migrating zone of the upper pair was thought to be ceramides containing fatty acid moieties with 22-24 carbon atoms. The slightly slower migrating zone of the upper pair contained the ceramides with shorter fatty acids in the 16-18 carbon range. The lower pair of zones consisted of ceramides with hydroxy fatty acids. The slower moving zone of this lower pair contained hydroxy fatty acids of 16-18 carbons. The faster-moving zone contained hydroxy acids with 22-24 carbons.

When zone 4, which was eluted from the preparative TLC plate, was subjected to TLC using 95:5 C:M, at least six separate components were observed, using the vanillin phosphoric acid spray reagent. Several of these zones appeared as blue spots; others were violet. The results can be seen in Figure II.

The eluate from zone 4 was separated using TLC solvent systems indicated in Figure II. The best chromatography results of a ceramide
Figure II. Solvents for Separation of Components from Zone 4

The left spot on each plate is ceramide standard and the right spot is the eluate of zone 4 from the 24:7:1 C:M:W preparative TLC. The plates were removed from the solvent when the front reached the top of the silicic acid. The solvent systems were, reading from left to right, 8:2 ethylacetate:benzene, 95:5 chloroform:methanol, ethylacetate, and ether. The lower right spot on the plate developed with 95:5 C:M is actually made up of three zones.
standard were obtained with 95:5 C: M. In all systems, both the standard and the lipids from zone 4 streaked.

GLC of Lipids

The lipid extract from the Florisil columns was treated with BSTFA-1% TMCS. By programming the gas chromatograph from 230° to 300°C, 31 components could be seen. The major peaks appeared above 272°C. Ceramide standard, silanized in this manner, gave peaks in the same region. Gas chromatography of the lipids from zone 4 also showed peaks with the same retention times as components from ceramide standards. The tentative conclusion was that some of the material in zone 4 (the material of interest) was probably ceramide. Further means of identification were necessary. The absence of cholesterol as a major component in the lipids from the Florisil columns was confirmed by gas chromatography.

Samuelsson\textsuperscript{14, 19, 20} reported the GLC separation of ceramides as di-o-trimethylsilyl ether derivatives using hexamethyldisilazane: TMCS pyridine as the silylating reagent. The structures were confirmed using the LKB 9000 gas chromatograph-mass spectrometer.

Horning\textsuperscript{2} converted sphingomyelins directly to the respective di-o-trimethylsilyl ceramide using BSTFA or bis-trimethylsilylacetamide (BSA). Higher yields were obtained by heating the sphingomyelins in phenyl ether, eliminating the phosphoryl choline moiety, and then silylating.
Identification of Ceramides by Mass Spectrometry

A sample of silylated lipids from the Florisil columns gave 13 peaks by temperature-programming the GLC-mass spectrometer from 200-280°C. Although the resolution was poorer and the relative peak sizes were somewhat different on the GLC-mass spectrometer, the peaks could tentatively be related to the ceramide peaks seen by GLC on the F & M 402. These spectra were very difficult to count accurately over approximately 500 mass units. Identification of ceramides was possible by matching the lower molecular weight ion fragments to the major mass fragments of synthetic ceramides found by Samuelsson.\textsuperscript{14,19} Mass spectra of human aorta ceramides containing fatty acids 24:1 and 23:0 are shown in Figure III.

The fragments with m/e values of 311 and 426 are present as major ion fragments in all of the ceramides in the sphingosine series.\textsuperscript{14} A characteristic m/e 311 peak is represented by fragment A (Figure IV) and the m/e 426 peak by the M-(B+1) fragment. The fatty acid is determined by the M-A and the M-(A-73) peaks. In each case, a peak believed to be the molecular ion minus 15 mass units (M-15) was present, but could not be accurately counted.

Identifications of four ceramides from the zone 4 lipids were made with the gas chromatograph-mass spectrometer. These ceramides contained fatty acid moieties 24:0, 24:1, 23:0, and 16:0. Other peaks present were presumed to be ceramides; however, adequate spectra were not obtained for positive identification.
The low molecular weight peaks, the isotope peaks, and background peaks have not been included.
Figure IV. Fragmentation Scheme of a Ceramide

\begin{align*}
\text{1,3-di-o-trimethylsilyl-N-tricosanoyl sphingosine (M = 779)}
\end{align*}

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Fragment Lost</th>
<th>M.W. of Ion</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-D</td>
<td></td>
<td>311</td>
</tr>
<tr>
<td>M-(B+1)</td>
<td></td>
<td>426</td>
</tr>
<tr>
<td>M-15</td>
<td>-CH_3</td>
<td>764</td>
</tr>
<tr>
<td>M-90</td>
<td>-CH_2OTMS</td>
<td>676</td>
</tr>
<tr>
<td>M-103</td>
<td></td>
<td>689</td>
</tr>
<tr>
<td>M-(A-73)</td>
<td></td>
<td>541</td>
</tr>
<tr>
<td>M-A</td>
<td></td>
<td>468</td>
</tr>
</tbody>
</table>
Fatty Acid Composition of Zone 4

The fatty acid compositions of zone 4 and the cerebrosides of human aorta are shown in Table II.

Table II. Fatty Acid Compositions of Zone 4 and the Cerebroside Zone

<table>
<thead>
<tr>
<th>Fatty Acids from Methanolysis</th>
<th>Ceramides** A</th>
<th>B</th>
<th>Cerebrosides A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>26</td>
<td>39</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>18:0</td>
<td>7</td>
<td>7</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>18:1</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>20:0</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>22:0</td>
<td>11</td>
<td>10</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>23:0</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>24:0</td>
<td>28</td>
<td>17</td>
<td>33</td>
<td>31</td>
</tr>
<tr>
<td>24:1</td>
<td>15</td>
<td>17</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>25:0</td>
<td>-</td>
<td>-</td>
<td>5</td>
<td>-</td>
</tr>
</tbody>
</table>

A and B are from respective batches of human aorta.

* The peaks containing the unsaturated fatty acid may also contain traces of fatty acids having more than one double bond.

** Zone 4 may contain some transesterifiable fatty acids other than those coming from ceramides.

Fatty acid compositions of ceramides and cerebrosides from the aorta extracts do not appear to be similar. Identities of the fatty acid methyl esters were confirmed by comparing their retention times with standards, and by observing the molecular ions on the gas chromatograph-mass spectrometer. As determined by mass spectrometry,
other components coming off the gas chromatograph after the 25:0 methyl ester were not related to the fatty acids.

While only four ceramides were identified by mass spectrometry, other peaks were observed on GLC and were tentatively identified as ceramides. The relative sizes and retention times of peaks identified as ceramides were consistent with the relative amounts of fatty acids shown in Table II. It is probable that ceramides were present with each of the fatty acids reported in Table II and that they were the major components yielding fatty acid esters from zone 4.

Possible Origin of Ceramides Found in Human Aorta Lipids

Aorta plaque lipids may be derived from plasma, or by synthesis in situ, or both. Finding the relative proportions of individual lipids from these sources has been a difficult problem. The filtration theory, one of many theories for plaque formation, states that elevated levels of plasma lipids cause lipid precipitation or accumulation on the aortic intima, eventually leading to atherosclerosis. Another theory assumes that the lipids are synthesized by the aortic intima, and that atherosclerosis is the result of derangement of lipid metabolism in the aorta. Since ceramide is an intermediate in the synthesis and catabolism of cerebrosides, sulfatides, sphingomyelins, and probably gangliosides, it seems probable that ceramides should be present in tissues containing any of these compounds. Since the fatty acid composition of these ceramides is somewhat different than aorta cerebrosides analyzed from the same
batch of aortas, it would seem unlikely that these are derived solely from cerebrosides by a simple hydrolysis mechanism. Sphingomyelin is the only other ceramide containing lipid reported in aorta.

Böttcher studied the sphingomyelin fatty acid compositions in human aorta wall in various stages of atherosclerosis. The fatty acid composition of sphingomyelins from aortas classified as stage III atherosclerosis was similar to that found for ceramides (zone 4) from these atherosclerotic aortas. Since relatively large amounts of sphingomyelin are present in aorta lipids (approximately two-fifths of the total phospholipid in human atherosclerotic aorta in sphingomyelin), it is conceivable that ceramides are intermediates in the metabolism of sphingomyelins in aorta.

Panganamala found, in atherosclerotic human aortas, considerable amounts of sphingolipids which contained long-chain bases other than sphingosine. Considerable amounts of the long-chain bases were sphingodienines and sphinganine. Ceramides containing these other long-chain base analogues are not easily separated by GLC from the ceramides containing sphingosine. Long-chain bases other than sphingosine were not detected by mass spectrometry.

Identification of Glycerol Ethers by Mass Spectrometry

Mass spectra were obtained for other components present in zone 4. Most of the material that was not ceramide-related came off the GLC prior to ceramides. Three peaks, which became apparent only after silylation of zone 4, were tentatively identified by their mass spectra as disilylated glyceryl ethers (alkoxyglycerols): chimyl
alcohol, batyl alcohol, and an alcohol having two mass units less than batyl alcohol. The mass spectrum of silylated T-octadecyl-glycerol (batyl alcohol) is shown in Figure V.

The most prominent peak in the mass spectrum of these three glyceryl ethers had an m/e of 205. This is probably the same m/e 205 ion reported by DeJongh for several silylated sugars. The m/e 205 peak was assigned the structure of TMS-OCH$_2$-CH$_2$-OTMSi, which indicated geminal primary and secondary alcohols. The peaks at m/e 473, 398, and 308 probably represent M-15, M-90, and M-180 fragments. The identifications of chimer and batyl alcohols were confirmed by mass spectra of the TMS derivatives made from standards of these alcohols. Molecular ions were not observed for the saturated alcohols, but a weak molecular ion was present for the 18:1-1 glyceryl ether. The position of the double bond in the 18:1-1 glyceryl ether was not determined with certainty. Both alkyl and alk-1-enyl glyceryl ethers have been identified in human fat. Hallgren and Larsson found that approximately 70% of the glyceryl ethers isolated from human tissues were the 16:0-1, 18:0-1, and 18:1-1 ethers. Glyceryl ethers, if present, were below the detection limits in human red blood cells. It is likely that glyceryl ethers are sometimes present in blood plasma, since they are well absorbed and are found in many foods, particularly fish oils. Tuna and Mangold reported the presence of diacyl glyceryl ethers in human aorta, but did not find the free alcohol.
Figure V. Mass Spectra of Silylated Batyl Alcohol and an Unidentified Component.

Batyl Alcohol

Peak II (not identified)

The low molecular weight peaks and the isotope peaks are not shown.
The presence of these glyceryl ethers in zone 4 agrees with the four conditions previously outlined (see INTRODUCTION). They migrate in the 24:7:1 C:M:W TLC system with OH-ceramides, are stable to alkaline methanolysis, and might behave similarly to carbohydrates in reactions with adjacent hydroxyls.

Mass Spectra of Other Materials from Zone Four

A peak coming off the gas chromatograph after the trimethylsilyl-chimyl alcohol peak (peak II) was clearly resolved, but not identified. The mass spectrum is shown in Figure V. The m/e 387 peak was 73 mass units less than the m/e 460 peak. This probably represents the loss of a trimethylsilyl group. Assuming that there was only one TMS in the molecule, the molecular weight of the original compound was 388.

An additional GLC peak having a retention time about three times as long as the 24:0 ceramide had major peaks at m/e 311 and 426, this indicated a sphingosine-containing compound. Due to the low intensity of these peaks, the higher mass peaks could not be detected and identification was not made.

Since the recovery of the components from zone 4 was not studied, no attempt was made to quantitate amounts of any of these components. Other components were present in zone 4, but were not adaptable to analysis by GLC and mass spectrometry, or were not in sufficient quantities to be positively identified.
Sugar Analysis

No sugar was detected on analysis of zones 4 and 5. The discrepancy with earlier work cannot be adequately explained, and was not pursued further in this study.³
BIBLIOGRAPHY


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SUMMARY

A crude glycolipid fraction was isolated from atherosclerotic human aortas by the Folch extraction, Florisil chromatography, mild alkaline methanolysis, and further Florisil chromatography. TLC of these lipids gave a zone migrating ahead of cerebrosides and having an unknown composition (zone 4). Analysis of the TMS ethers of the TLC zone showed multiple components by GLC. These were subsequently analyzed by mass spectrometry.

Ceramides containing fatty acids 16:0, 23:0, 24:1, and 24:0 were identified by GLC-mass spectrometry. Acid methanolysis of zone 4 showed methyl esters from ceramides containing fatty acids 16:0, 18:0, 18:1, 20:0, 22:0, 23:0, 24:0, 24:1, and 25:0. These were present in amounts similar to fatty acids found in aortic sphingomyelin, suggesting a metabolic relationship between ceramide and sphingomyelin.

Batyl alcohol, chimyl alcohol, and an 18:1-1 glyceryl ether were identified as components present in this zone by gas chromatography-mass spectrometry of their silyl ethers. Other unidentified components may have been present.
VITA

The author was born and reared on an Iowa farm. He received his B.S. degree from McPherson College, McPherson, Kansas, and attended the University of Michigan and Eastern Michigan University while working as a technician at the University of Michigan. For the past eight years, he has been a research assistant at The Upjohn Company and, more recently, a part-time student in chemistry at Western Michigan University.