THE CHEMISTRY AND ANALYSIS OF SPHINGOLIPIDS
IN NATURAL PRODUCTS

by

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Being a progress report on research carried out
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1976
To the best of my knowledge this thesis contains no material which has been accepted for the award of any other degree or diploma in any University and contains no copy or paraphrase of material previously published or written by another person except where due reference is made in the text.

E. George
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ABSTRACT

Methods used for the analysis of both synthetic and natural sphingolipids and their components are described.

Analytical data is given on ceramides derived from bovine-brain cerebrosides, and on the sphingolipids derived from milk from a predominantly Fresian herd. The major fatty acids in brain sphingolipids are 24:0, h24:0, 22:0, h22:0, 18:0 and h18:0; 24:1 is a less major component in brain cerebrosides, but a major component in brain sphingomyelin. The milk sphingolipids comprise mainly sphingomyelin, ceramide glucoside, and ceramide lactoside. All three sphingolipids contain both non-hydroxy and hydroxy fatty acids, and their compositions are similar; 23:0, 24:0, 22:0 and 16:0 (and the corresponding hydroxy fatty acids) predominate, with 16:0 (or h16:0) and 24:1 (or h24:1) present to a lesser degree.

The separation of diastereoisomeric ceramides by TLC on borate-impregnated silica gel, and by GLC, is described. Synthetic ceramides are prepared for this work by direct coupling of long chain base and fatty acid in the presence of a carbodiimide. Side-reaction products produced by this method are analysed, accounting for previously unknown TLC spots present in the crude preparations.

Finally the analysis of human serum lipids by spectrodensitometry using phosphomolybdic acid is described. The methods are applied to serums obtained from 68 apparently healthy adults, and quantitative results given for ceramides, monoglycerides, and cholesterol (free and total).
CHAPTER 1

1.1. Introduction: Thin-layer Chromatography

Thin-layer chromatography (TLC) has become, over the past two decades, one of the main analytical methods used in lipid research. It is a very simple technique, and is sensitive, rapid and economical. Besides the fractionation of complex natural lipid mixtures, which is perhaps its principal use, the purity of lipid preparations can be assessed by TLC as well as the identification of lipid structures by chromatographic mobility. It can also be used for monitoring column separations and extractions. The subject has been reviewed in much detail by Marinetti (1967), who discussed the TLC analysis (also column and paper chromatography) of neutral glycerides and fatty acids, phosphatides and glycolipids, and sphingosine and related compounds. The work in this thesis uses TLC as its main analytical tool, and references important in the analysis of milk lipids, ceramides and polar lipids are given throughout the thesis.

Materials, methods

TLC plates were prepared by slurrying silica gel (from E. Merck AG, Germany) with water in a stoppered flask by vigorous shaking for five minutes, and applying to glass plates by means of a commercial applicator. The plates were 20 cm wide, and varied in length from 5 cm to 100 cm (for analytical work, 5 cm, 10 cm, or 20 cm x 20 cm plates were used, and for preparative work, 40 cm or 100 cm x 20 cm plates were used). For analytical plates (250μ thick) the slurry composition was 30 g silica gel : 58 ml water (or solution containing silver nitrate, borax, etc.); for preparative plates (1 mm thick) the slurry
composition was 100 g silica gel : 175 ml water. After preparation, the plates were either dried for 30 minutes at 85°C in the case of ceramide analyses, or activated for 30 minutes at 120°C in the case of phosphatide analyses. The plates were then stored in cabinets containing silica gel blue dessicant until used. Silver nitrate impregnated plates darkened quickly on exposure to light, and had to be used immediately, or stored in the dark in an atmosphere of dry nitrogen.

Most analytical and preparative analysis was carried out in either of the two solvents:


The TLC tanks were lined with filter paper to saturate the atmosphere with the solvent, and the tanks allowed to stand at least one hour before beginning chromatography experiments. Fresh solvent was necessary at the start of each week, as the more volatile components of the solvent mixtures were lost, and impurities were liberated into the solvent from the adsorbent.

For detection of the lipids on analytical plates the general method was to spray a fine mist of 50% sulphuric acid containing 0.5% \( \text{K}_2\text{Cr}_2\text{O}_7 \) onto the plate under the fume hood and char the lipids by heating the plate in an oven for 30 min. at 350°C. Permanent recording of plates was done on a Polaroid overhead camera using Polaroid 107 black and white film or 108 colour film.

For detection of lipid bands on preparative plates, the method
used was to spray the plate lightly with an aqueous solution of easin
(0.1% w/v), and view the plate at 254 nm. The fluorescence was
quenched in the presence of even minute amounts of lipids which showed
up as blue bands on an iridescent green background; concentrated
lipids occurred as yellow bands. The portion of silica gel containing
the lipids was scraped off the plates, and the lipids extracted with
chloroform-methanol (2:1). The indicator was completely removed by
washing the extract with water.

1.2. Gas-liquid Chromatography

The literature available on the applications and theory of gas-
liquid chromatography (GLC) to the analysis of lipids and related
compounds is immense. One of the best reviews is that given by
Marinetti, who deals with, besides thin-layer and column chromatography,
the GC analysis of glycerides, alkoxy lipids, fatty acids and derivat-
ives, long-chain aldehydes, sphingosines, carbohydrates in glycolipids,
and nitrogen bases derived from lipids.

Analysis of fatty acids (FA)

GLC was carried out on a Phillips PV 4000 dual column gas
chromatograph, which was modified to take glass columns (required for
later work with sphingosines analyses) with Pye-Unicam injection heads
for on-column injection. The columns were 5 mm OD x 2 m glass coils,
silanized by treatment with 5% trimethylchlorosilane (TMCS) in toluene.
For the column packing, 3% SE-30 on 100-120 mesh Gas Chrom Q gave good
separation of saturated and monounsaturated normal and hydroxy fatty
acid methyl esters (FAME). Polyunsaturated methyl esters separated
on a column containing a polar liquid phase, EGSS-X. The silicone
phase, SE-30, as well as OV-1 (both stable to 350°C) will both prove
to be useful in later work for the analysis of the other constituents of sphingolipids i.e. long chain bases and sugars, as well as molecular species.

Operating conditions for the analyses of FAME in this thesis, on the SE-30 columns are: temperature programming, 160° to 310°C/min; N₂ flow rate, 20 ml/min; H₂ flow rate, 20 ml/min; air flow rate 250 ml/min; injector temperature, 310°C; chart speed, 0.25 inch/min; attenuation, 5 to 50 x 10². Columns were conditioned 3 days at maximum temperature (350°C) before initial use.

Pure standards (usually >99% purity) of fatty acids or FAME available for reference were: 6:0, 8:0, 10:0, 12:0, 14:0, 16:0, and 18:0(a); 17:0, 19:0, 20:0, 21:0, 22:0, 23:0, and 24:0(b); 18:1, 18:2, 18:3, 20:1, 22:1, and 24:1(b); h18:0, h20:0, h22:0, h24:0 and h24:1(a); h16:0 and h26:0(c)*. Other fatty acids were determined from compounds of known fatty acid composition e.g. beef brain sphingomyelin and OHFA cerebrosides². Fatty acids were converted to methyl esters prior to GLC by refluxing the FA in methanol containing 5% sulphuric acid, and extracting the FAME with hexane. Hydroxy FA were either silylated or acetylated and analysed as trimethylsilyl ethers or acetoxy methyl esters.

The retention times of normal and hydroxy-FAME relative to methyl stearate are tabulated (Table 1:2-1), and graphically illustrated (Fig. 1:2-1). It can be seen that the retention time is directly proportional to the carbon number for both the normal and OH-FAME, and the graphs may be extrapolated to determine with a good degree of accuracy the retention times of higher molecular weight FAME.

(a) Obtained from Applied Science Laboratories, State College, P.A., USA.
(b) Obtained from Sigma Chemical Co., St. Louis, Mo., USA.
(c) Obtained from Fluka AG., Switzerland.
* Chain length and degree of unsaturation are indicated by numerals, i.e. 18:1 is an 18 - carbon monounsaturated acid; h indicates a 2 - hydroxy acid.
Quantitation of the FAME was made simple by the fact that peak height was found to be proportional to concentration for each FAME, and that the peak heights were equal for equal quantities of FAME injected onto the column. Thus the relationship used for area measurement, concentration \( \propto \text{area} = \text{peak height} \times \text{width at half peak height}, \) reduced to concentration \( \propto \text{peak height} \) in most cases. The integrator on the recorder was used when necessary.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Normal</th>
<th>Hydroxy (TMSi ether)</th>
<th>Acetoxy</th>
</tr>
</thead>
<tbody>
<tr>
<td>12:0</td>
<td>0.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14:0</td>
<td>0.47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>0.73</td>
<td>1.02</td>
<td>0.19</td>
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<tr>
<td>18:0</td>
<td>1.00</td>
<td>1.29</td>
<td>0.38</td>
</tr>
<tr>
<td>20:0</td>
<td>1.27</td>
<td>1.55</td>
<td>0.59</td>
</tr>
<tr>
<td>22:0</td>
<td>1.53</td>
<td>1.78</td>
<td>0.86</td>
</tr>
<tr>
<td>24:0</td>
<td>1.78</td>
<td>2.01</td>
<td>1.13</td>
</tr>
<tr>
<td>26:0</td>
<td></td>
<td>2.23</td>
<td>1.34</td>
</tr>
<tr>
<td>18:1</td>
<td>1.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2</td>
<td>0.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:3</td>
<td>0.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:1</td>
<td>1.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22:1</td>
<td>1.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24:1</td>
<td>1.75</td>
<td>2.20</td>
<td></td>
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</table>
In the past few years applications of high pressure liquid chromatography (HPLC) have increased significantly. The technique has been widely employed in the analysis of nucleotides and drugs but relatively little work has been done in the area of lipid analysis. This can be attributed partly to deficiencies in detection devices currently available for use. The UV photometer is the most widely used detector; however, most lipids have no useful absorption in the UV range. This limitation is being overcome, as methods for the formation of UV absorbing derivatives are being developed\textsuperscript{2-4}.

The theory, instrumentation, and some applications is covered by Kirkland,\textsuperscript{5} Snyder\textsuperscript{6} and Brown.\textsuperscript{7} This section presents a short review

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Fig. 1.2-1. Retentions of fatty acid methyl esters (FAME), relative to 18:0.
of the HPLC analyses of lipids, and some analytical methods used on the Pye Unicam LMC2 liquid chromatograph.

1.3.1. Non-polar lipids and fatty acids

The advantages of monitoring lipid separation on a silicic acid column with a liquid chromatograph has been presented by Worth and MacLeod. These authors achieved separation between cholesteryl esters, triglycerides, free fatty acids, free cholesterol, diglycerides, and monoglycerides (from blood plasma), using increasing concentrations of ether in petroleum ether for elution.

Stolyhwo and Privett have obtained very good separation of reference mixtures of non-polar lipids, using slightly increased pressures (ca. 20 psi), and continuous changes of pentane, ethyl ether and chloroform. Their column contained a superficially porous support 'Corasil 11"; this type of support (other examples: 'Zipax' and surface etched glass beads) has been shown by Kirkland to give better results than the completely porous materials such as silicic acid and 'Porosil', which have a higher resistance to mass transfer resulting in low column efficiency.

Gel permeation chromatography has been the method of choice for the analysis of non-polar lipids when high pressures (ca. 10-40 atm) are involved.

Bombough et al. used a series of 500 Å Styrage columns and tetrahydrofuran eluent to separate tricaproin, tricaprin, trimyristin and tristearin. Poragel (60-100 Å) has been used on a preparative scale for the separation of triarachidin, trimyristin and trilaurin. Lawrence has used 200 Å Poragel, and 5% aqueous acetone as the eluent to separate monopalmitin, palmitic acid, 1,3-dipalmitin,
cholesterol, and tripalmitin.

The most recent review of HPLC of fatty acids is given by Cooper and Anders, 1975; no other references could be found in the literature. In almost all cases, controlled surface porocity supports were used, mainly the Corasils. The fatty acids were converted to UV-absorbing derivatives. Thus, Politzer et al. (Anal. Letters 6:539, 1973) separated palmitic, heptadecanoic, and stearic acids as their benzyl esters; Cooper and Anders reported the analysis of unsaturated C18 and C20 fatty acids as their 2-naphthacyl esters; Schofield (J. Amer. Oil Chem. Soc. 52:36, 1975) resolved linseed esters (i.e. 18:3, 18:2, 18:1, 18:0 and 16:0 methyl esters), and reported good separations with soy-bean, safflower, corn and olive esters; Mikes et al. used silver nitrate impregnated Corasil 11 columns to separate stearic, oleic, and elaidic acid methyl esters.

Shorter chain fatty acids have also been analysed by HPLC. Mowery and Juvet used a reversed phase Bondapak C18/Corasil column and water as eluent to separate 8:0, 9:0 and 10:0 fatty acids; Takata and Muto used an ion exchange column to separate maleic, fumaric, and C1 to C6 fatty acids.

Hydroxy fatty acids have not been investigated, to my knowledge.

1.3.2. Polar Lipids

Stolyhwo and Privett obtained good separation of reference mixtures of polar lipids on Corasil 11 using a continuous gradient change of chloroform to ammoniacal methanol; beef-brain cerebrosides (2 main peaks, 4 minor components), cholesterol, phosphatidyl ethanolamine, phosphatidyl choline, and beef brain sphingomyelins (2 peaks) were resolved.
Jungalwala et al.\(^4\) separated phospholipids containing primary amino groups (i.e. phosphatidylethanolamine, ethanolamine plasmalogens, and phosphatidyl serine from rat tissue) as their p-biphenylcarboxyl derivatives on a Micropak SI-10 (a special silica gel, av. size 10 \(\mu\)m) column using dichloromethane-methanol - 15 M \(\text{NH}_3\) mixtures for elution.

Lairon et al.\(^{18}\) has separated glycerophosphorylcholine, phosphorylcholine, glycerophosphate, and orthophosphate on Dowex 1-X4 anion exchange resin, for the purpose of studying the degradation of bile lecithins.

Particular applications of HPLC to the analysis of sphingolipids has been reported. Evans and McCluer\(^{19}\) separated mono-, di-, tri-, and tetra- glycosyl ceramides, as their benzoyl derivatives on a Zipax column using gradient elution of 0.2 to 0.75% methanol in hexanes and detection of 254 nm. The glycolipids were extracted from human plasma and erythrocytes, and the plasma glycolipid profile of patients with Fabry's and Gaucher's disease was studied.

Sugita et al.\(^3\) and Iwamori and Moser\(^{21}\) have demonstrated elevated amounts of free ceramide in Faber's disease urine and tissues by HPLC on Zipax\(^3\) (average size 27 \(\mu\)m), or Micropak SI-10\(^{21}\) using 0.05% methanol in pentane or 2.5% ethylacetate in hexane. The ceramides were analysed as benzoyl derivatives, and good resolution of ceramide derivatives containing nonhydroxy or hydroxy fatty acids was obtained.

Iwamori and coworkers\(^{22}\) analysed 3-ketosphingolipids (ceramides, cerebrosides, sulphatides, sphingomyelin) by HPLC on Micropak SI-10 at 254 nm; normal and hydroxy fatty acid sphingolipids were separated. The authors used the method to determine ceramide levels in Faber's disease patients, and cerebroside and sulphatide levels in metachromic leukodistrophy brains.
Recently good preparative separation of neutral glycosphingolipids from human erythrocytes was achieved by HPLC using totally porous silica spheres, "Iatrobeads" (Ando et al., 1976\textsuperscript{23}). The solvent was a linear gradient of a CHCl\textsubscript{3}/CH\textsubscript{3}OH/H\textsubscript{2}O mixture, and the glycolipids were detected by anthrone-sulphuric acid reagent. Compared to the irregularly shaped 100 mesh silicic acid commercially available (Mallinckrodt) the solvent flow through the spherical Iatrobeads was rapid, and elution bands migrated with minimal diffusion and hence better resolution. Ceramide dihexoside, ceramide trihexoside, and two ceramide tetrasaccharides (globoside 1 and paragloboside) were completely resolved on Iatrobeads. Furthermore, the ceramide dihexoside and ceramide trihexoside both appeared as two overlapping fractions, corresponding to molecular species containing either C\textsubscript{16}:0, or C\textsubscript{24}:0 plus C\textsubscript{24}:1 as the major fatty acids (hydroxy fatty acids were found absent in the glycolipids of human erythrocytes).

1.3.3. HPLC using the Pye Unicam LMC2 Instrument

A diagrammatic representation of the Pye Unicam LMC2 liquid chromatography equipped with a moving-wire flame ionization detector (FID) and LKB Uvicord (type 4701A) ultraviolet detector is shown by figure 1.3-1. A moving, high precision, stainless steel wire passes from a feed spool through a tube of alumino-silicate glass in the cleaner portion of an oxidizer/cleaner oven. The tube is flushed with CO\textsubscript{2}-free air, and the solute is oxidized off the wire to carbon dioxide and water at a temperature of 500 to 800°C. These gases are sucked up into a molecular entrainer, and swept out into a reaction chamber by a stream of hydrogen mixed with an inert gas such as nitrogen. The specially prepared catalyst in the chamber and a temperature of 350°C causes the formation of methane which passes into
the FID operating at a temperature of 175°C. The resulting signal, directly related to the number of carbons in the original compound, is suitably amplified on a normal 1 mV or 10 mV potentiometric recorder.

The analytical column (2.6 mm ID x 500 mm thick glass tubing contained in a metal cylinder) was dry-packed in silica gel (Merck) prepared as outlined in Fig. 1.3-2.

1.3.4. Applications

As an introduction to the use of the instrument, some highly coloured UV-absorbing derivatives were prepared and chromatographed on the silica gel column. The derivatives were esters prepared by refluxing p-phenylazobenzoyl chloride and a slight excess of an
alcohol in pyridine, as described by Woolfolk et. al. They were purified by alumina column chromatography and recrystallization from ethanol (details are given in the author's M.Sc. Qual. Report, 1973). Hexane-diethyl ether (9:1) was the eluting solvent, and a column inlet pressure of 20 kg/cm² gave a flow rate of 1.4 ml/min. 100 µg of ester in 10 µl of CCl₄ was injected onto the column of each analysis. Results, illustrated in Fig. 1.3-3, show that octyl p-phenylazobenzoate is clearly separated from the cholesteryl ester, but the cetyl ester overlaps the other two.

Benzoylated cerebrosides and ceramides (beef brain) prepared with
Fig. 2. High pressure liquid chromatograph of synthetic esters of p-phenylazobenzoyl chloride. 100 µg of each ester in 10 µl CCl₄ was injected and eluted with hexane-diethyl ether 9:1; (a) Cholesteryl phenylazobenzoate; (b) Palmityl phenylazobenzoate; (c) Octyl phenylazobenzoate

benzoic anhydride as described by McCluer and Evans,24 were each observed to separate into two fractions on the silica gel prepared as described in Fig. 1.3-2. The eluting solvent was 0.25% CH₃OH in hexane, with flow rate 1.4 ml/min and inlet pressure 20 kg/cm². These fractions were apparently the non-hydroxy and hydroxy fatty acid ceramides3 or cerebrosides.¹⁹
1.4. Methods for the analysis of natural lipids by spectro-densitometry

Within the spectrum of non-polar lipids of natural products, the analysis of cholesterol, 1,2- and 1,3- diglycerides, triglycerides and cholesteryl esters by densitometry is made relatively simple by the fact that these lipids are completely separated on TLC plates. However, the analysis of minute amounts of free ceramides is made difficult by the occurrence of large quantities of free fatty acids (FFA) and monoglycerides which migrate to the same general area on the plate (FFA move slightly faster than monoglycerides). An initial attempt to overcome this problem made use of the cis-glycol complexing effects of borate and arsenite ions. TLC plates, impregnated with from 1 to 10% Na₂B₄O₇·10H₂O or NaAsO₂, were used, and the following observations made. Arsenite retarded the motion of FFA (on 10% plates it remained on the origin), while α-monoglycerides moved upwards level with the non-hydroxy-fatty-acid ceramides (non-OHFA ceramides). Borate had the reverse effect: monoglycerides appeared just below the OHFA ceramides, while FFA moved level with the non-OHFA ceramides. Borate also has a complexing effect on trans-double bonds, and ceramides containing sphing-4-enine and sphinganine are separated.

For the analysis of free ceramides in plant products, these complexing agents would rise to an even more complex situation, as borate retards N-acyl phytosphingosines so that they migrate with the cerebrosides, while arsenite has the reverse effect, causing them to move close to the solvent front.

1.4.1. Removal of FFA from naturally occurring lipid mixtures

Two simple methods were developed for the 100% removal of the FFA mixture present in all naturally occurring lipid extracts.
1. Sodium carbonate may be applied as a narrow band (cf. 0.5 to 1 cm) immediately before the origin on a previously prepared TLC plate by spraying a 10% aqueous solution between a pair of thin flat glass plates until the silica is saturated. The plate is then dried or activated and used for TLC as are normal plates. Photograph 1.4 illustrates this technique, showing the removal of FFA from a milk lipid extract (lane 4), and from a mixture of monoglycerides, previously isolated from milk by preparative chromatography (lane 3). The effect of the carbonate band on other lipids was also tested, using 20 cm x 20 cm plates of which only 10 cm was treated; the untreated portion of the plate served as a control. The following lipids migrated quantitatively on their normal $R_f$ values through the alkaline barrier: tripalmitin, triolein, 1,2-dipalmitin, 1,3-dipalmitin, monopalmitin; monoolein, cholesterol, cholesteryl stearate, methyl oleate, ceramides, cerebrosides, phosphatidyl ethanolamines, phosphatidyl serines, lecithins, sulphatides, sphingomyelins and lysolecithins.

2. Diazomethane reacts quantitatively with FFA to form their methyl esters. An ethereal solution of $\mathrm{CH}_2\mathrm{N}_2$ was prepared by distilling a reaction mixture consisting of 2 g of p-tolysulphonylmethylnitrosamide dissolved in 30 ml of ether, and 10 ml of 4% ethanolic KOH; the nitrosamide was previously prepared from p-toluenesulphonyl chloride and methylvamine (Vogel). The $\mathrm{CH}_2\mathrm{N}_2$ solution is added to an ether-methanol (9:1) solution of the lipids until the yellow colour persists and $\mathrm{N}_2$ ceases to be liberated (normally, carotenoids, yellow in colour, are present in naturally occurring lipids, so that excess $\mathrm{CH}_2\mathrm{N}_2$ may be added). The mixture is then left at room temperature for 15 minutes, and the solvent evaporated in a stream of nitrogen; more ethyl ether is added and the evaporation continued until excess
CH$_2$N$_2$ is removed. The lipid residue is then dissolved in chloroform; TLC analysis shows the absence of FFA, but the presence of methyl esters at the solvent front. All lipids previously mentioned in the carbonate method, with the exception of phosphatidyl ethanolamine, seem to be unaffected by the CH$_2$N$_2$ treatment as judged by TLC.

1.4.2. Spectrodensitometry of lipids on thin-layer plates using phosphomolybdic acid

The quantitative analysis of individual lipid components isolated from a complex mixture by TLC is made rapid and sensitive by spectrodensitometric methods. Of decisive importance in the accuracy of the analysis are factors such as purity, thickness and degree of moisture of the adsorbent, the process used to apply the reagent to the chromatoplate, the locating reagent and the shape and demarcation of the spots as well as the distribution of components within the spot. The thickness and degree of moisture become insignificant factors if commercially prepared plates (e.g. Merck) are used, and if adequate storage facilities are available (cabinets containing silica gel blue dessicant were found suitable). The shape and demarcation of the spots can be controlled largely by the use of TLC tanks lined with filter paper so as to saturate the atmosphere within the TLC solvent. The time for complete saturation of the TLC tanks has been found to be of the order of 2-3 hours.

The processes which have been used to apply reagents to TLC plates are as follows: incorporation into the adsorbent before preparation of the TLC plates, spraying after TLC, and dipping after TLC. The advantages of incorporation compared to spraying are described by Touchstone et. al., who analysed steroids by densitometry on thin-layer plates impregnated with either sulphuric
acid, ammonium bisulphate, or phosphomolybdic acid. The method found most practical in this laboratory for the analysis of serum or milk lipids was that of Chedid and coworkers as follows.

Commercially available silica gel G TLC plates (supplied by E. Merck, Germany), 20 cm x 20 cm with either glass or transparent Mylar backings, were used. Each plate was washed by dipping the edge into chloroform-methanol (2:1) in a TLC chamber and allowing the solvent to ascend until the solvent front reaches the upper edge. Na$_2$CO$_3$ barriers (0.5 cm wide) were applied just above the origin and the plates were then dried at 90°C in an oven for 30 min.

Chloroform solutions of lipid standards or natural mixtures, of known concentration, were applied evenly to the plate over 1.0 cm widths and 1.5 cm apart, 2 cm from the bottom edge, by means of a micro-syringe. The plates were then developed in a solvent suitable to separate non-polar lipids; the usual solvent, chloroform-carbon-tetrachloride-methanol-water (50:50:10:0.5), was used. After drying, the plates were immersed evenly and slowly in a solution consisting of 5 g of phosphomolybdic acid in 70 ml of water, 25 ml of ethanol, and 5 ml of 70% HClO$_4$. The excess reagent was quickly removed with blotting paper, and the plates placed in an oven for 20 min exactly at 85°C; unsaturated lipids reduce the yellow phosphomolybdic acid to blue phosphomolybdate under these conditions. The plates were cooled for 10 min, and scanned on a Hilger Watts scanning densitometer.

1.4.3. Discussion

Results obtained by the above method were found to be reproducible from plate to plate. Neither the reagent on the plates nor the colour of the spots deteriorated after 1-2 days provided the plates were overlaid with a clean glass plate and stored in the dark;
after several weeks, however, the plates protected with glass gradually assumed a blue colour, while plates exposed to the atmosphere lost their blue spots and returned to the yellow colour of phosphomolybdic acid (presumably reoxidation).

Standard calibration curves for free cholesterol (from Fluka AG), non-OHFA ceramides (from Sigma Chem. Co.) and monoglycerides (approx 90% pure, from bovine milk) are illustrated in Fig. 1.4. As can be seen, the method is quite sensitive (0.05 μg cholesterol, 0.1 μg ceramide, and 0.5 μg monoglyceride). Neither of the curves for cholesterol or ceramides have the relationship area = kC^B (C = concentration), which was shown to be the case for lipids charred with sulphuric acid derived from (NH₄)_2SO₄ incorporated in the plates (Castellani et al., 1975).

The density of the spots vary greatly for the same concentration of different lipids, by comparison of the curves on Fig. 1.4. This indicates that other factors may be involved in the reduction of the phosphomolybdic acid besides the number of double bonds per mole of lipid; this remains to be studied. It was shown that a completely saturated monoglyceride, α-monopalmitin, did not reduce the phosphomolybdic acid at all neither when chromatographed separately, nor when in the presence of natural monoglyceride or OHFA ceramide mixtures. Thus hydroxyl and ester groups have no effect on the reagent under the conditions described. Tripalmitin and stearic acid showed no reaction either. Cholesterol is very simply analysed as it is in a single component, well separated from other lipids on TLC, and can be obtained commercially with purity >99%. However, for the analysis of, for instance, serum ceramides or monoglycerides in a complex lipid mixture, it would be desirable to have lipid standards of approximately the same composition.
Another factor which has not been discussed is the possible loss of some of the surface lipid into the staining reagent. This should be negligible for non-polar lipids, but the method may prove unsuitable for the analysis of the more polar phosphatides for this reason.
Fig. 1.4. Densitometry of lipids using phosphomolybdic acid
2.1. The Analysis of Koch Light Ceramides

Free ceramides are manufactured by the Koch Light Chemical Company from ox-brain cerebrosides by the elegant procedure of Carter et al.\textsuperscript{35} i.e. oxidation of the galactose moiety with periodate, followed by mild reduction, and acidic hydrolysis of the resulting acetal. The ceramides separate into four major bands on a TLC plate; these bands vary in intensity from batch to batch (photograph 2.1-1). The identities of the two fast-moving bands occurring at \( R_f \) 0.60 and \( R_f \) 0.40 are known from previous work and literature available;\textsuperscript{25} they are non-hydroxy or normal fatty acid ceramides, and hydroxy fatty acid ceramides, respectively. The identities of the two slow-moving bands occurring at \( R_f \) 0.20 and \( R_f \) 0.14 are unknown, however. It is the purpose of this section to present evidence for the nature of the unknown bands, and to give a complete analysis of the long-chain base and fatty acid constituents.

2.1.1. Preparation of acetyl sphingosines derived from ceramides

100 mg of Koch-Light ceramides from different batches were separated into four crude bands on a preparative 1 metre plate, and each band purified twice on analytical plates to produce 25 mg of normal fatty acid ceramides, 20 mg of hydroxy fatty acid ceramides, 25 mg of band 3 and 18 mg of band 4. 8 mg of each ceramide band was hydrolysed with 4 ml of 1 M KOH\textsuperscript{36} in methanol at 75°C in teflon-lined screw-capped tubes (by-product formation and stereochemistry changes are minimised using alkaline rather than acidic hydrolysis). The fast-
moving ceramide bands were completely hydrolysed overnight, but the slow-moving bands were only 30-40% hydrolysed and required 3 days for 90-95% hydrolysis. The long-chain bases were recovered by adding 4 mls of water and 8 mls of ethyl ether and partitioning the bases into the ether layer; the remaining solutions of fatty soaps were set aside for fatty acid analysis at a later stage. To prevent degradation of the unstable sphingosine bases either by TLC or storage, the N-acetyl derivatives were prepared immediately by selective N-acylation with acetic anhydride in methanol, as follows. 3 mg of long-chain base derived from each ceramide band was dissolved in 200 μl of methanol and the solution mixed with 10 μl of acetic anhydride. After standing overnight at room temperature, 20 mls of water were added and the mixture was chilled in ice for several hours. The white solid was centrifuged, washed with water twice, and dried in vacuo over calcium chloride to yield about 3 mg of acetylated material (>90% yield). The product was purified once on a 20 cm analytical TLC plate to remove 0-acetylated products.

Totally acetylated sphingosines were prepared by treating 0.5 mg of free or N-acetyl long-chain base with 100 μl of acetic anhydride in 0.5 ml of anhydrous pyridine overnight; the excess anhydride was destroyed with 100 μl of methanol. 8 ml of ether was added, and the solution washed three times with 2 ml of 1N hydrochloric acid, three times with 2 ml of 5% sodium bicarbonate solution, and three times with water. The solution was dried and the ether removed to yield the crude totally acetylated derivative; nearly 100% was recovered.

For TLC comparison of the acetylated compounds, prepared as above, standards were made up from sphingosine, dihydrosphingosine, and phytosphingosine; sphingosine was initially freed from its sulphate salt by TLC with chloroform-methanol-water 49:49:2. A thin-layer chromatogram of the N-acetyl derivatives (photograph 2.1-2)
1,2 and 3: three different samples of ceramides, manufactured by Koch Light at different times (batch nos. 4 3209, 56936, unknown, respectively); 4: K.L. cerebrosides; 5: Cholesterol.

Photo 2.1-2

DN: N-acetyl, D,L racemic dihydrosphingosine;
SN: N-acetyl D,L-erythro sphingosine (impure);
C: K.L. ceramides
shows no difference in the $R_f$ value (0.2) of N-acetylsphingosine and its saturated analogue. The unsaturated compound was not purified and two pairs of spots are observed in the upper portion of the TLC plate. These would most likely be diacetyl (i.e. di-Ac $O^1N^2$, $N^2O^3$, $O^1O^3$) and triacetyl derivatives of sphingosine. The existence of pairs of spots rather than single spots due to the threo- N-acetylsphingosine seems unlikely since many authors, particularly H.E. Carter, have established the erythro- stereochemistry of naturally occurring sphingolipids; it is noteworthy that free threo-sphingosine may be the intermediate in the enzymatic synthesis of sphingomyelin.\textsuperscript{43} Also, sphingosine is isolated by alkaline hydrolysis of naturally occurring sphingolipids, excluding the possibility of inversion known to occur to N-acyl-1,2-amino alcohols during hydrolysis by acidic reagents.\textsuperscript{38}

The N-acetyl sphingosine was purified twice by preparative TLC to produce 10 mg of a white solid that separated into two spots very close together by TLC on analytical plates, thus accounting for the elongated spot on photograph 2.1-2. These spots were better separated on TLC plates impregnated with either borate or silver ions; the less-intense, faster-moving spot is due to N-acetyl-dihydrosphingosine, which mass spectroscopy showed to be present to the extent of 7% in the sphingosine. O-methyl ethers, which are formed through acidic methanolysis of sphingolipids\textsuperscript{44} and which have similar $R_f$ values as the free sphingosine,\textsuperscript{39} were found absent by analysis of the mass spectrum. An infrared spectrum of the N-acetyl derivative in chloroform shows peaks characteristic of N-acyl sphingosine LCB at 3410-3270 cm\textsuperscript{-1} (NH, OH stretch), 3080, 1640, 1545, 2190, 1085, 1040 and 940 cm\textsuperscript{-1} (the latter due to C-H out-of-plane deformation due to trans double bond). There is negligible ester carbonly absorption in the 1740 cm\textsuperscript{-1} region. (See Fig. 2.1-1).
Fig. 2.1-l. Infrared spectrum of N-acetyl-D,L-erythro sphingosine (in CHCl₃)
C: K.L. Ceramides; 1,2,3 and 4: Acetylated L.C.B. derived from K.L. ceramides 1,2,3 and 4, respectively (subscripts N and T indicate N-acetylation or Total-acetylation); D: Dihydrosphingosine; P: Phytosphingosine.
N-acetyl dihydrosphingosine (photograph 2.1-2, $D_N$) showed a similar TLC pattern to the unsaturated compound before purification. The dihydrosphingosine used to prepare the derivative was a synthetic preparation, and thus the derivative consisted of four possible isomers: $D,L$-erythro N-acetyl dihydrosphingosine (upper spot), and $D,L$-threo N-acetyl dihydrosphingosine. The compound was synthesised by two methods, both $N$-selective: (a) direct coupling of the long-chain base and acetic acid in the presence of a mixed carbodiimide\textsuperscript{45} (b) acetic anhydride in methanol.\textsuperscript{40} Both methods gave high yields (>95%) of the N-acetyl derivative which gave rise to similar TLC patterns. Its melting point was 110-113°C, intermediate between the erythro and threo isomers.\textsuperscript{16}

The N-acetyl and totally acetylated derivatives of the L.C.B. derived from K.L. ceramides are represented on photograph 2.1-3 compared to acetylated dihydrosphingosine (the sphingosine derivatives have the same $R_f$ values) and acetylated phytosphingosine. The latter derivative is relevant in that even though phytosphingosine has not yet been observed in brain sphingolipids, the presence of the extra hydroxyl group on carbon 4 considerably alters the $R_f$ values of its derivatives. In fact the positions of the $N$- or tetra-acetyl-phytosphingosine on the TLC plates are only slightly higher than the acetylated L.C.B. derived from K.L. slow ceramides. These results indicate that the difference in the chemical nature of the normal and slow ceramides lies not in the fatty acid moiety (though hydroxy fatty acids account for the difference between ceramide bands 1 and 2) but the long-chain base moiety. Also, the L.C.B. derived from the slow ceramides probably contain extra hydroxyl groups, as indicated by: (a) T.L.C. behaviour of their $N$- or totally-acetylated derivatives; (b) infrared spectra of their purified naturally occurring or N-acetyl derivatives compared to the fast ceramide derivatives show a much
stronger C-O stretch at 1040-1050 cm\(^{-1}\), indicating an increase in primary alcohol content. The C-H out-of-plane deformation at 960 cm\(^{-1}\) for the \textit{trans} double bond is present in all derivatives for both fast and slow ceramides.

2.1.2. **Mass spectroscopic determination of L.C.B. derived from K.L. ceramides**

The composition of each of the L.C.B. fractions derived from the K.L. ceramides was determined by mass spectroscopy of the trimethylsilyl ethers\(^{47}\) of their N-acetyl derivatives. Briefly, the results of the mass spectra indicate that both normal and slow ceramides contain mainly C\(_{18}\) sphingosine with smaller amounts of C\(_{18}\) dihydro-sphingosine, but the terminal carbon of the slow ceramides is linked through the oxygen to a polyol group:

\[
\begin{align*}
\text{O} & \quad \text{C} & \quad \text{CH}_2\text{OH} \\
\text{CH}_2\text{OH} & \quad \text{C} & \quad \text{OH} \\
\end{align*}
\]

i.e. the slow-moving bands of K.L. ceramides are the acetals obtained on periodate oxidation of cerebrosides followed by sodium borohydride reduction of the resulting dialdehydes.

The mass spectra of the di-O-TMSi-N-acetyl sphingosines derived from K.L. ceramides 1 (Fig. 2.1-2) and 2 may be easily identified by comparison with mass spectra of standards, (Figs. 2.1-3 and 2.1-4) and by reference to the literature concerning the same derivatives.\(^{40,48}\) Major ions at m/e 311 and m/4 174 result from cleavage between C\(_2\) and C\(_3\) for the sphingosine derivative, with charge retention on the L.C.B. fragment (M-d)* or fatty acid fragment (M-a) respectively; for the dihydrosphingosine derivative (M-d) occurs at m/e 313. For homologous

* Refer to mass spectra for the meaning of the symbols M,a,b,c,d
Fig. 2.1-2. Mass spectrum of the di-O-trimethylsilyl-N-acetyl sphingosines derived from Koch Light normal fatty acid ceramides (no. 1. band)
Fig. 2.1-3. Partial mass spectrum of di-O-TMSi-N-acetylphosphatidylcholine derived from sphingosine sulphate supplied by Koch Light Chemical Co. (see Fig. 2.1-2 for meanings of symbols).
Fig. 2.1-4. Partial mass spectrum of di-O-TMSi-N-acetyldihydrosphingosine, derived from D,L-rac.dihydrosphingosine (synthetic). See Fig. 2.1-2 for meanings of symbols.
L.C.B., ions corresponding to (m-d) could not be found. Thus from the relative intensities of these peaks ceramides 1 and 2 contain 22% and 14% dihydrosphingosine respectively, and the remainder sphingosine. Other L.C.B. fragments occur at \( M-(b+1) = m/e \ 426 \) for loss of the acetamido group plus a neighbouring hydrogen, and \( M-(b+1+90) = m/e \ 336 \) for the loss of the terminal trimethylsilanol as well (a very small peak only).

The molecular weight of sphingosine is clearly indicated by the presence of peaks at \( m/e \ 470 \) (\( M-15 \)) for loss of a methyl group from one of the trimethylsilyl residues, \( m/e \ 395 \) (\( M-90 \)) for loss of trimethylsilanol, and \( m/e \ 382 \) (\( M-103 \)) for loss of the terminal \( \text{CH}_2\text{OTMSi} \) group.

Cleavage between \( C_2 \) and \( C_3 \) with transfer of the trimethylsilyl group to the nitrogen was first postulated for ceramides by Samuelsson. The same kind of ions are observed in the mass spectra of N-acetyl-trimethylsiloxylated bases, and the following process would seem probable:

\[
\begin{align*}
\text{R-CH-CH-CH}_2\text{OTMSi} & \quad \rightarrow \quad \text{CH-CH}_2\text{OTMSi}^+ \\
\text{O}^+\cdot\text{NH} & \quad \rightarrow \quad \text{TMSi}^+\cdot\text{NH} \\
\text{TMSi} & \quad \rightarrow \quad \text{COCH}_3
\end{align*}
\]

\[
\begin{align*}
m/e \ 247 & \quad \text{(m-a+73)} \\
-\text{TMSiOH} & \\
\text{CH-CH}_2 & \quad \rightarrow \quad m/e \ 157 \\
\text{TMSi}^+ & \quad \text{(M-a+73-90)} \\
\text{COCH}_3 & \quad \rightarrow \\
\end{align*}
\]
The ions occurring at m/e 243 and m/e 217 result through a loss of acetamide, plus $\text{CH}_3(\text{CH}_2)_{12}$ for sphingosine (i.e. $(M-(b+1+e))$, or $\text{CH}_3(\text{CH}_2)_{14}$ for dihydrosphingosine (i.e. $M-(b+1+c)$), respectively. The latter ion is observed to be much more intense than the former.

The mass spectrum of the siloxylated N-acetyl L.C.B. derived from K.L. ceramide 3 (Fig. 2.1-5) has prominent peaks at m/e 311 and m/e 313, indicating $\text{C}_{18}$ sphingosine and $\text{C}_{18}$ dihydrosphingosine as for the normal ceramides; the ratio is 93:7 in this case. Some other fragmentation modes are represented by the dashed lines on the diagram of the proposed structure for this molecule. Two important mass spectral mechanisms to account for some of these ions involves the separate consideration of the effect of the radical and positive ion sites, as proposed by McLafferty. If $A$ represents the sphingosine part of the molecule, and $B$ the polyol part, then the two mechanisms are as follows:

$$A-\text{CH}_2\text{-}\text{O}\text{-}B \quad \rightarrow \quad A^\dagger + \text{CH}_2 = O - B \quad \ldots \ldots \ (i)$$

$$A-\text{CH}_2\text{-}O^\dagger - B \quad \rightarrow \quad A - \text{CH}_2 - O^\dagger + B^\dagger \quad \ldots \ldots \ (ii)$$

Most of the major ions observed are due to mechanism (ii), i.e. transfer of an electron pair adjacent either $A$ or $B$ to the positive charge on the oxygen atom. Thus the ions at m/e 396 and m/e 398 for the sphingosine and dihydrosphingosine derivatives respectively are probably due to the following process:

$$\text{R - CH - CH - CH}_2 - O^\dagger - \text{CH} \quad \rightarrow \quad \text{R - CH - CH - CH}_2 + O - \text{CH}$$

$$\begin{array}{c}
\text{TMSiO} \\
\text{NH} \\
\text{COCH}_3
\end{array} \quad \rightarrow \quad \begin{array}{c}
\text{TMSiO} \\
\text{NH} \\
\text{COCH}_3
\end{array}$$

m/e 396 or 398
Fig. 2.1-5. Mass spectrum of the tetra-O-TMSi-N-acetylsphingosines derived from Koch Light ceramide band 3.
The ratio of peak heights between the saturated and unsaturated derivatives is much higher for these ions than for the ratio of peak heights for the (M-d) ions. However, differences such as this occur otherwise between derivatives of the two bases. Other ions due to mechanism (ii) with charge retention on the polyol fragments occur at m/e 351, m/e 219 and m/e 262 (the latter due to loss of trimethylsilanol from the ion at m/e 351) as demonstrated in the proposed structure. An ion expected at m/e 452 for cleavage between C₂ and C₃ and charge retention on the acid fragment does not appear, but the ions at m/e 306 and m/e 216 may result from secondary rearrangement losses of two trimethylsilyl ions, followed by a trimethylsilanol molecule, respectively.

Peaks resulting from the possible presence of 1,3,4-tri-O-trimethylsilyl-N-acetylphytosphingosine could not be found in this mass spectrum, by comparison with the mass spectrum of a pure sample.

2.1.3. Periodate oxidation of cerebrosides followed by conversion to ceramides

To check the nature of K.L. ceramides, cerebrosides (both Sigma and K.L.) were separated into fractions, subjected to oxidation with sodium metaperiodate, followed by reduction with sodium borohydride, and hydrolysis with cold methanolic hydrochloric acid. Reaction products at various stages were checked by TLC.

150 mg of cerebrosides (grade II, Sigma) were separated by preparative TLC into 25 mg of non-hydroxy-fatty acid cerebrosides (ceb-N), 85 mg of hydroxy-fatty acid cerebrosides (ceb-OH), 26 mg of sulphatides, and about 4 mg of a yellow-coloured lipid of about the same polarity as ceramide. The purified glycosides are represented on photograph 2.1-4 compared to total K.L. and Sigma cerebrosides; the
latter are observed to contain a much greater amount of hydroxy fatty
acid cerebrosides and sulphatides compared to K.L. cerebrosides.

5 mg of cerebroside, purified once more by TLC, were oxidised
with finely powdered sodium metaperiodate suspended in either
anhydrous pyridine, or aqueous methanol and stirring vigorously
overnight. On oxidation of a cerebroside fraction that gives a
single TLC spot, two spots are obtained for the dialdehyde (photograph
2.1-5). The non-OH-FA dialdehydes move faster than the OH-FA
dialdehydes, as may be expected. Reduction of the dialdehydes to the
polyols was carried out in slightly alkaline methanol at room
temperature for four hours. The alkaline solutions were made just
acidic with HCl to destroy excess NaBH₄, and the polyols extracted
into chloroform using the Folch partition method. Results are shown
on photograph 2.1-6, showing the presence of slow ceramides, with
some fast ceramides probably derived from the slightly acidic
conditions before extraction.

Finally, the polyol fractions derived from the cerebrosides, as
well as purified samples of K.L. ceramides 3 and 4, were treated with
cold 0.6 N methanolic HCl for 1 hour, after which the solutions were
made just alkaline (NaOH) and extracted with chloroform by the Folch
partition method. TLC of the resulting hydrolysis products is
demonstrated by photograph 2.1-7. Here it is clearly seen that K.L.
ceramide 3 is the polyol derived from the normal fatty acid cerebro-
sides and hydrolyses to normal fatty acid ceramides; similarly K.L.
ceramide 4 is the polyol derived from the hydroxy-fatty acid
cerebrosides, and hydrolyses to the hydroxy-fatty acid ceramides.
The dialdehydes are also easily hydrolysed under the same conditions,
but give rise to incomplete separation of bands (column 4), probably
because of their greater reactivity under basic or acidic conditions.

1: K.L. ceramides; 2, 3 and 4: Dialdehydes obtained by IO₄⁻ oxidation of total K.L., non-OH-FA Sigma and OH-FA Sigma cerebrosides, respectively; 5: IO₄⁻ oxidation products of Sigma sulphatides; 6: Cholesterol.
C: K.L. ceramides; 1, 2 and 3: Polyols derived from total cerebrosides, non-OH-FA cerebrosides, and OH-FA cerebrosides, respectively, by IO₄⁻ oxidation followed by reduction; 4: IO₄⁻ oxidation products of cerebrosides.

C: K.L. Ceramides; 1, 2 and 3: Ceramides derived from total cerebrosides (K.L.), non-OH-FA cerebrosides and OH-FA cerebrosides (Sigma), respectively, by IO₄⁻ oxidation followed by reduction and hydrolysis; 4: Ceramides derived from K.L. cerebrosides by IO₄⁻ oxidation plus hydrolysis; 5: Cerebrosides, treated mildly with acid; 6 and 7: Ceramides derived from K.L. "slow" ceramides 3 and 4, respectively, by treatment with cold 0.5 N methanolic HCl.
The lower dialdehyde spot obtained on $\text{IO}_4^-$ oxidation of both ceb-N and ceb-OH seems to be unaffected by the reduction and hydrolysis treatments.

Sulphatides, subjected to the same treatment as the cerebrosides, were not affected by the final hydrolysis treatment; in fact the bond linking the degraded sugar sulphate was found just as resistant as the original glycosidic bond before oxidation to the acetal i.e. requiring hot acidic conditions for hydrolysis.

2.1.4. Analysis of fatty acids

The aqueous-methanolic solution of fatty soaps remaining after the long-chain bases had been extracted was acidified strongly with 6 N HCl and the mixture extracted with ethyl ether. The methyl esters were prepared$^{53}$ by adding 4 ml of 1 N methanolic hydrogen chloride to the fatty acid residue in a 12 ml teflon-lined screw-capped tube, and heating at 75°C for 2 hours. 0.5 ml of water was added, and the methyl esters extracted with petroleum ether 40-60. They were then purified by thin-layer chromatography on 20 cm analytical plates developed with ether-hexane (4:6 by vol.) to 10 cm height, followed by development with benzene to the top of the plate. Normal fatty acid methyl esters were located in the upper section of the plate ($R_f \approx 0.85$), and hydroxy fatty acid methyl esters in the lower section of the plate ($R_f \approx 0.50$). The esters were extracted from the silica gel with diethyl ether for GLC analysis.

Gas-liquid chromatographic analyses were performed on 3% SE-30 columns identical to those described in 1:2, conditions, flow rates, etc. are also the same. Hydroxy fatty acid methyl esters were analysed as trimethylsilyl ethers, prepared as described in 1.2.
The results of the analyses of Koch Light ceramides are tabulated in Table 2.1-1, and for comparison results (Table 2.1-2) are also given for the fatty acid analyses of ceramides derived from bovine brain cerebrosides (Applied Science Laboratories), and ceramides derived from bovine brain sphingomyelin by enzymatic hydrolysis.36

Results of fatty acid analysis

Both non-hydroxy or normal fatty acid and hydroxy fatty acid compositions of Koch Light and Applied Science ceramides include a wide range of long-chain acids from 12 to 30 carbons, both saturated and monounsaturated. The non-hydroxy fatty acids present in highest concentration are, in decreasing order: lignoceric, behenic and stearic acids followed by nervonic, tricosanoic, hexacosaenoic and palmitic acids on about the same level. A similar order is observed for the corresponding hydroxy fatty acids. These results do contradict the fatty acid analyses given by O'Brien and Rouser54 for beef brain cerebrosides, who state the most important fatty acids to be, in decreasing order, 24:1 (or h24:0), and 24:0 (or h24:1 and h18:0); 22:0 (or h22:0) was a minor constituent. The reason for the difference cannot be explained, at present; one fact is that it is not due to the process of chemically degrading the cerebroside to ceramide, as Hammarstrom55 obtained results in good agreement with O'Brien's and Rouser's when characterizing ceramides derived from brain cerebrosides.

The results obtained for sphingomyelin (Table 2.2), however, are very similar to those of O'Brien and Rouser54, the most important fatty acids being 18:0, 24:1 and 24:0 in decreasing order.
<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Ceramide 1 Normal</th>
<th>Ceramide 3</th>
<th>Ceramide 2 Hydroxy</th>
<th>Ceramide 4 Hydroxy</th>
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<tr>
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<td>0.1</td>
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<tr>
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<td>-</td>
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<td>-</td>
</tr>
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<td>0.1</td>
</tr>
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<td>11.9</td>
<td>10.7</td>
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<td>0.5</td>
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<td>5.1</td>
<td>3.5</td>
<td>5.8</td>
<td>1.0</td>
</tr>
<tr>
<td>27:0</td>
<td>0.2</td>
<td>4.3</td>
<td>0.7</td>
<td>6.9</td>
</tr>
<tr>
<td>27:1</td>
<td>0.4</td>
<td>0.1</td>
<td>tr</td>
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</tr>
<tr>
<td>28:0</td>
<td>0.1</td>
<td>0.6</td>
<td>0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>28:1</td>
<td>0.3</td>
<td>2.6</td>
<td>0.6</td>
<td>4.0</td>
</tr>
<tr>
<td>29:0</td>
<td>tr</td>
<td>1.5</td>
<td>0.5</td>
<td>2.6</td>
</tr>
<tr>
<td>29:1</td>
<td>-</td>
<td>0.3</td>
<td>tr</td>
<td>0.5</td>
</tr>
<tr>
<td>30:0</td>
<td>tr</td>
<td>1.9</td>
<td>0.3</td>
<td>1.5</td>
</tr>
<tr>
<td>Fatty acid</td>
<td>Sphingomyelin (Normal Only)</td>
<td>Ceramides, ex-bovine brain cerebrosides Normal</td>
<td>Hydroxy</td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>-----------------------------</td>
<td>-----------------------------------------------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ceramides, ex-bovine brain cerebrosides Normal</td>
<td>Hydroxy</td>
<td></td>
</tr>
<tr>
<td>12:0</td>
<td>0.1</td>
<td>-</td>
<td>tr</td>
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<tr>
<td>14:0</td>
<td>0.1</td>
<td>tr</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>15:0</td>
<td>0.4</td>
<td>0.1</td>
<td>-</td>
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</tr>
<tr>
<td>16:0</td>
<td>2.9</td>
<td>0.3</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>16:1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>17:0</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>18:0</td>
<td>36.8</td>
<td>10.7</td>
<td>16.3</td>
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<tr>
<td>18:1</td>
<td>0.5</td>
<td>1.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2</td>
<td>0.3</td>
<td>-</td>
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<td>19:0</td>
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<td>0.4</td>
<td>0.4</td>
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<tr>
<td>20:0</td>
<td>0.7</td>
<td>2.4</td>
<td>1.6</td>
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<tr>
<td>20:1</td>
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<td>-</td>
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<tr>
<td>21:0</td>
<td>0.1</td>
<td>0.4</td>
<td>0.6</td>
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<tr>
<td>21:1</td>
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</tr>
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<td>22:0</td>
<td>2.8</td>
<td>15.3</td>
<td>13.6</td>
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<tr>
<td>22:1</td>
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<td>0.8</td>
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<tr>
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<tr>
<td>23:1</td>
<td>1.1</td>
<td>1.8</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>24:0</td>
<td>8.8</td>
<td>30.1</td>
<td>37.5</td>
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</tr>
<tr>
<td>24:1</td>
<td>26.1</td>
<td>15.4</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
<td>25:0</td>
<td>2.4</td>
<td>2.5</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>25:1</td>
<td>4.8</td>
<td>2.3</td>
<td>1.3</td>
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<tr>
<td>26:0</td>
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<td>2.8</td>
<td>4.0</td>
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</tr>
<tr>
<td>26:1</td>
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<td>4.2</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>27:0</td>
<td>0.3</td>
<td>0.5</td>
<td>0.4</td>
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</tr>
<tr>
<td>27:1</td>
<td>0.6</td>
<td>0.5</td>
<td>0.5</td>
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<tr>
<td>28:0</td>
<td>0.3</td>
<td>0.9</td>
<td>0.8</td>
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</tr>
<tr>
<td>28:1</td>
<td>0.5</td>
<td>-</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>29:0</td>
<td>-</td>
<td>-</td>
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<tr>
<td>29:1</td>
<td>-</td>
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<td></td>
</tr>
<tr>
<td>30:0</td>
<td>tr</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

* Ceramides containing hydroxy or non-hydroxy fatty acids supplied by Applied Science Laboratories.
A result which is worth mentioning, and which can be seen by comparing the columns of Table 2.1-1, is that on the average no particular cerebroside molecule is preferentially degraded chemically to the corresponding ceramide molecule i.e. the polyols representing ceramide 3 and ceramide 4 have similar fatty acid compositions as their corresponding hydrolysis products, ceramide 1 and ceramide 2. However, the polyols do seem to contain a large proportion of very long chain fatty acids (C27 to C30), which are not usually present to such an extent in naturally occurring sphingolipids.

2.2. Analysis of Ceramides: Separation of Diastereoisomers

Of all the natural sphingolipids examined so far, the D-erythro configuration has been established for sphingosines, and D-ribo for phytosphingosines. However, the threeo-isomers play an important part in the chemistry and metabolism of sphingolipids. The two known biosynthetic pathways for sphingomyelin, i.e., transfer of phosphorylcholine moiety of CDP choline to ceramide, and N-acylation of sphingosylphosphorylcholine, require threeo-sphingosine as a precursor; in the second pathway, erythrosphingosylphosphorylcholine is also an active lipid acceptor. Also, hydrolysis of sphingolipids by acidic reagents causes inversion of the secondary hydroxyl group, thus producing threeo-sphingosines. Welsh has shown that high yields of threeo-1,2-amino alcohols are formed by treatment of their erythro-isomers with acids.

The separation of free diastereoisomeric sphingosine bases has been accomplished on silica-gel thin-layer plates, and as their dinitrophenyl derivatives of borate-impregnated plates. The following report describes methods of separation of erythro- and threo-N-acyl sphinganines (ceramides), by TLC or GLC. Also noted is the
complete separation of ceramides containing a trans-double bond in the long-chain base (i.e. N-acyl sphing-4t-enines), and ceramides containing sphinganine; this effect has been observed before, but not studied in any detail.

2.2.1. Methods

Ceramides, containing D,L-erythro sphing-4-enine (freed from its sulphate salt with 1 equivalent of methanolic NaOH, then excess BaCl₂), sphinganine (DL racemic-, DL erythro-, or DL threo-), and saturated, unsaturated, or saturated-2-hydroxy fatty acids, were prepared by direct coupling of the long-chain base with the fatty acid in the presence of a mixed carbodiimide. After alkaline and neutral washes, the crude ceramide mixtures were treated with diazomethane in 10% methanolic ether for 15 minutes, and purified on plain or 10% borate impregnated silica gel TLC plates (40 cm x 20 cm x 250 μ); the TLC solvent was CHCl₃-CCl₄-CH₃OH-H₂O (50:50:11:0.5). Hydroxy fatty acid ceramides were purified as the 2-acetyl derivatives. The plates were then sprayed with 1% aqueous eosin, ceramide bands marked under the UV light, and the ceramides eluted from the silica gel with CHCl₃-CH₃OH (2:1). The dye was removed by adding ½ vol. of water and shaking vigorously, and the chloroform layer evaporated to dryness.

Unreacted long-chain base and fatty acid methyl esters were also recovered.

For GLC analysis the ceramides were converted to the trimethylsilyl ethers, as follows: To ca. 0.5 mg of ceramide dissolved in 100 μl of dry pyridine, 20 μl of hexamethyldisilizane and 10 μl of trimethylchlorosilane were added and the mixture left at room temperature for 30 minutes. The pyridine was removed under vacuo using an oil pump, and the residue dissolved in 200 μl of carbon
disulphide. Analyses were carried out on 3% SE-30 columns, with flow rates, etc. the same as described in 1.2. Programming conditions are given on the chromatograms.

2.2.2. TLC results

The TLC plate shown on photograph 2.2-1 illustrates the successful separation of fully saturated diastereoisomeric ceramides. The erythro- isomer (lane 3) moves ahead of the threo-isomer (lane 4), indicating the latter is more strongly complexed with the B$_4$O$_7$ anion. The erythro- isomer is also observed to move level with the non OHFA N-acyl sphinganines of naturally occurring ceramides, indicating they also have the erythro- configuration. If threo- N-acyl sphinganines did exist in natural isolated ceramides, they would be masked by the erythro- N-acyl sphing-4-enines, since they occur at about the same $R_f$ value.

Saturated isomeric OHFA ceramides also separate successfully (photograph 2.2-2). The ceramides were prepared from racemic 2-hydroxy hexacosanoic acid, and DL-racemic dihydrosphingosine. A total of three plates were required to separate the eight possible isomers into four racemic pairs. The left plate on photograph 2.2-2 shows the separation, on a plain silica gel plate, of ceramides into two overlapping pairs; the upper pair contains L-h16:0 (lane 3), and the lower pair contains D-h16:0 (lane 4). (The separation of OHFA ceramide diastereoisomers, whose stereochemistry differs only in asymmetric centre of the OHFA, has been reported before.\textsuperscript{25,45,68,69} Both diastereoisomeric pairs separate completely into single bands (erythro- or threo-) on borate impregnated silica gel, as illustrated by the right plate of photograph 2.2-2.)
1. Ceramides derived from bovine brain cerebrosides;
2. Diastereoisomeric N-behenoyl D,L-sphinganines (prepared from DL racemic dihydrosphingosine);
3. N-behenoyl D,L-erythro sphinganine (prepared from D,L-erythro dihydrosphingosine);
4. N-behenoyl D,L-threo sphinganine (prepared from D,L-threo dihydrosphingosine; some erythro-dihydrosphingosine is present as an impurity).

Thin-layer chromatography of ceramides.
Separation of ceramides containing sphing-4t-enine and sphinganine on borate impregnated silica gel.

<table>
<thead>
<tr>
<th>Photo 2.2-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Separation of ceramides of same carbon number, differing only in stereochemistry and number of double bonds. The natural ceramides of lane 1 were derived from bovine serum brain sphingomyelins by enzymolysis using phospholipase C; the major fatty acids present are 18:0 (40%), 24:1 (24.6%) and 24:0 (10.7%).</td>
</tr>
</tbody>
</table>
Borate-impregnated plates may also be used to separate ceramides, synthetic or naturally occurring, which differ in the long-chain base moiety; ceramides containing sphinganine move ahead of ceramides containing sphing-4t-enine (photograph 2.2-3). The allylic centre is somehow involved, because isomeric ceramides, differing only in the geometrical configuration of the double bond (i.e. cis- or trans-), occur at the same R<sub>f</sub> value as ceramides containing saturated fatty acids (photograph 2.2-4). An increase in cis- unsaturation slightly increases the mobility of the ceramides (lanes 3-6) on both borate-impregnated and ordinary silica gel plates.

Silver nitrate impregnated silica gel has been successfully used for the separation of ceramides containing cis- or trans- mono-unsaturated fatty acids; the silver ion did not distinguish between the sphinganine and sphing-4t-enine moieties.

2.2.3. GLC results

Gas-liquid chromatography also proved to be a useful technique for the identification of diastereoisomeric ceramides. Whether the sample was run through the columns isothermally or with temperature programming, ceramides (both non-OHFA and OHFA types) containing the erythro-configuration were well separated from their threo counterparts; the former always had the greatest retention time. Columns with 1% OV-1 as well as of 3% SE-30 were tested, and similar separations were observed; only the retention times were altered.

The chromatogram for D,L- h16:0 - D,L rac-d18:0 (Fig. 2.1) illustrates the above observations i.e. the separation of erythro- and threo- isomeric ceramides. However, with hydroxy fatty acid ceramides such as this one the configuration of the fatty acid affects the overall picture and more than two peaks are observed. A
Fig. 2.1. Gas-liquid chromatography of tri-O-trimethylsilyl ether derivatives of ceramides containing DL rac-sphinganine and racemic 2-hydroxyhexadecanoic acid. L or D indicate the configuration of the hydroxy fatty acid, while e or t indicate the configuration of the long-chain base (i.e. erythro or threo); thus De = 1,3,2'-tri-O-trimethylsilyl-N-(2'D-hydroxyhexadecanoyl) DL erythro-1,3-dihydroxy-2-aminoctadecane). Column: 3% SE-30 on 100-120 mesh Gas-Chrom Q; temperature programming of 270°C to 330°C @ 5°C/minute.
ceramide containing a racemic hydroxy fatty acid and threo- long-chain base are separated from each other, and both from their erythro-counterparts. However, a ceramide containing a racemic hydroxy fatty acid and erythro- long-chain base is represented by a single peak only; this latter result has been observed before by Hammarström, on a similar GLC column.

2.2.4. Discussion

Studies on the separation of diastereoisomers using borate impregnated TLC plates can be extended to include ceramides containing isomeric sphing-4-enine, and phytosphingosine bases, cerebrosides and sphingomyelins. The separation of erythro and threo- psychosines and sphingosylphosphorylcholines on ordinary gel plates has been reported before.

Arsenite and lead impregnated plates were also tested but these agents neither resolved ceramide diastereoisomers, nor separated sphinganine ceramides from sphingenine ceramides. Morris did, however, successfully separate isomeric polyhydroxy fatty acids on arsenite impregnated plates (better resolution was obtained using borate plates), but he found that silica gel layers impregnated with basic lead acetate, sodium metavanadate, or sodium molybdate resulted in migrations almost identical to those obtained on untreated silica gel.

2.3. Analysis of some Polar Lipids derived from Milk

The structure and composition of bovine milk triglycerides has been well documented. The polar lipids, which comprise about 1% of the total lipids, have been studied to a much lesser extent. Of the
total phospholipid fraction, the sphingolipids comprise about 30%, which is taken up as follows: sphingomyelin, 23%; ceramide clycosides, 6%; free ceramides, 0.006%; and gangliosides, 1-2%. The following chapter presents some analytical data on the sphingolipids derived from a predominantly Friesan herd (> 90% Friesan, 5% Jersey, traces of Guernsey and Hereford), and describes methods developed for the isolation of the polar lipids.

2.3.1. Preparation of crude, polar lipid fractions

1200 ml of fresh milk (containing about 50 g of lipid) was freeze-dried using an Edwards model 10 P freeze-drier; a Dynavac oil-pump could create a vacuum of 0.05 mm Hg in this unit, and 8 hours were required to completely dry 80 ml of milk spread as a thin film over the inside surface of a 1-litre conical flask. The dry milk powder (155 g), which now contained about 35% lipid, was treated four times with 900 ml of chloroform-methanol (2:1) each time, and the total combined extract partitioned by adding 0.2 volume (720 ml) of water. After standing overnight the chloroform layer was dried over anhydrous magnesium sulphate, and the solvent was removed under vacuo at 40°C.

To remove the neutral lipids a combination of the following two methods were found satisfactory.

(a) Partition between polar and non-polar solvents. The lipid residue (~ 50 g) was dissolved in 120 ml of the upper phase obtained when petroleum ether 40-60 is equilibrated with an equal volume of 87% ethanol. The upper phase was then extracted about twenty times with 40-50 ml each time of the lower phase. Monitoring of this process by TLC showed that after six extractions the phosho- and glyco-lipids (including cerebrosides) were completely partitioned into
the ethanol phase. Ceramides, and other lipids of similar polarity (e.g. free fatty acids, monoglycerides) are more soluble in the petroleum ether phase, and a further fourteen extractions were required to remove 95% of this fraction. The total extract was diluted with benzene and concentrated in vacuo to yield a lipid fraction containing about 30% phospholipids.

(b) Column chromatography. Several methods are available in the literature using a variety of adsorbents and solvents, but the following quick, simple method worked well when crude fractions were required. Silicic acid (Mallinckrodt 100-120 mesh powder) was pretreated by washing out the fines with water (advantages of, given by Galanos and Kapoulas\textsuperscript{81}), and activating at 120°C for 2 hours immediately before use. 100 g of the treated powder was slurried with 240 ml of diethyl ether, and the mixture used to prepare a column of dimensions 19 cm high x 3½ cm diameter. A column of dimensions of this order has been recommended (Rouser et. al., 1967\textsuperscript{1}) for the isolation of lipid classes from 1-2 g of a mixture.

The phospholipid fraction obtained in (a) above (1½ g) was dissolved in a small volume of diethyl ether, and applied to the top of the silicic acid column. The neutral lipids, mainly glycerides, cholesterol esters and some cholesterol were eluted (2-3 ml/min) with 400 ml of diethyl ether and discarded. The remainder of the cholesterol, along with ceramides, free fatty acids, monoglycerides and some phospholipids were eluted with 150 ml of chloroform followed by 100 ml of chloroform-methanol (9:1); the total eluate (fraction I) was set aside for further fractionation and analysis of individual components. The remainder of the lipids were eluted with a gradient of 10 to 100% methanol in chloroform, and the total eluate (fraction II) set aside for further analysis. The whole process was carried out using an automatic fraction collector, and was TLC-monitored, so that
any desired fraction could be taken.

2.3.2. Isolation of lipid classes from fraction I

Free fatty acids and monoglycerides were isolated in the nearly pure state by chromatography of the total lipid of fraction I on a 1 metre preparative TLC plate (solvent: CHCl₃-CCL₄-CH₃OH-H₂O (50:50:10:0.5)). The phospho- and glyco- lipids at the origin were recovered and added to fraction II. Free ceramides could not be detected on the TLC plate either by charring or by the amido test (Table 2.3-1). However, in the IR spectra of the crude fatty acid and monoglyceride fractions there appeared a weak absorption band at 1550 cm⁻¹, corresponding to the N-H deformation, but no C=O stretch for amides; only the strong C=O stretches for acid or ester above 1700 cm⁻¹. In subsequent purifications of the fatty acids and monoglycerides, by analytical TLC the 1550 cm⁻¹ band disappeared from the latter, but remained in the fatty acids. This would seem to indicate the presence of non-hydroxy fatty acid ceramides, but such compounds were not present in large enough amounts to be isolated after removal of the fatty acids as their methyl esters (i.e. after treatment with diazomethane).

The TLC positions of free fatty acids and monoglycerides in relation to other lipids are illustrated on photograph 2.3-1. The milk lipids (lane 3) were obtained from fresh milk by the method of Gloster and Fletcher, as follows: 1 ml of milk was shaken with 20 ml of chloroform-methanol (2:1), and allowed to stand 30 min before adding 4 ml of 0.1 M aqueous sodium chloride. The mixture was shaken, and the phases allowed to separate over several hours at 4°C before removing and evaporating the chloroform layer under vacuo. The lipid residue was dissolved in 0.25 ml of chloroform.
Thin-layer chromatogram of milk lipids.

1. Ceramides, ex-bovine brain cerebrosides;
2. Milk monoglycerides; 3. Total milk lipids;
4. Free fatty acids from milk; 5. Cholesterol, Solvent A.


**TABLE 2.3-1**

**Spray reagents used for the detection of lipids on TLC plates**

<table>
<thead>
<tr>
<th>Test</th>
<th>Reagents</th>
<th>Colour reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Amido (NH)</td>
<td>Spray with 2% t-butylhypochlorite in cyclohexane, dry 1 hour, then spray with 1% KI-starch.</td>
<td>Blue-black on white background</td>
</tr>
<tr>
<td>2. Ester</td>
<td>Spray with alkaline hydroxylamine (mix 1 g of NH₂OH·HCl in 9 ml of water with 2 g of NaOH in 8 ml of water), dry at 110°C for 10 min., then spray with acidic ferric nitrate (mix 1 g of Fe(NO₃)₃·9H₂O in 15 ml of water with 10 ml of acetic acid plus 1.5 ml of HCl).</td>
<td>Yellow on white background</td>
</tr>
<tr>
<td>3. Vicinal diol</td>
<td>Spray with 1% NaIO₄ until the plate is saturated, leave 10 min, and place in a tank saturated with SO₂ for 15 min. Then spray with Schiff's reagent (1% aqueous p-rosaniline hydrochloride decolorized with SO₂, and treated with charcoal powder), and replace in the tank.</td>
<td>Deep pink to blue on white background</td>
</tr>
<tr>
<td>4. Acid</td>
<td>Spray with 0.1% methanolic bromocresol green, to which just sufficient NaOH has been added to produce a blue colour.</td>
<td>Blue on yellow background</td>
</tr>
<tr>
<td>5. Free NH₂</td>
<td>Spray with 0.3% ninhydrin in acetone-lulidine (9:1).</td>
<td>Purple-mauve on white background</td>
</tr>
<tr>
<td>6. Phosphate</td>
<td>Spray with the following reagent: Shake for 30 min a mixture containing 2 g of ammonium molybdate in 16 ml of water, 8 ml of conc. HCl, and 2 ml of Hg, and filter (solution (a)). To 1 g of ammonium molybdate in 8 ml of water add 40 ml of conc. H₂SO₄, followed by solution (a). Cool, and dilute to 200 ml with water.</td>
<td>Blue on white background</td>
</tr>
<tr>
<td>7. Glycoside</td>
<td>Spray with 10% (v/v) H₂SO₄ solution, followed by 1% (w/v) anthrone in benzene. Heat at 100% for 10-15 min.</td>
<td>Blue-green on white background</td>
</tr>
</tbody>
</table>
2.3.3. Identification and analysis of the free fatty acids

The fatty acid band (82 mg) was identified by its similar action to oleic acid on TLC plates, positive acid test (Table 2.3-1), and practically identical infrared spectrum (Fig. 2.3-1) as oleic acid. Absorption bands at 3000 ~ 2400, 1710, 1280 and 920 cm\(^{-1}\) indicate a carboxylic acid dimer, and the band at 3000 cm\(^{-1}\) indicates unsaturation. Analysis of the fatty acids by GLC on 3% SE-30 show the following: oleic acid, 84.3%; palmitic acid; 14.0%, myristic acid 1.7%. These are the chief acids of milk triglycerides, in the same order.

2.3.4. Identification and analysis of the monoglycerides

The monoglycerides (37 mg) were identified by their similar action to \(\alpha\)-monopalmitin on both plain and borate-impregnated TLC plates, and positive ester and vicinal diol tests (Table 2.3-1). The infrared spectrum (Fig. 2.3-2) was similar to \(\alpha\)-monopalmitin, with OH stretching frequency between 3600 and 3200 cm\(^{-1}\), and C-O stretches at 1100 cm\(^{-1}\) and 1050 cm\(^{-1}\) for primary (1\(^{\circ}\)) and secondary (2\(^{\circ}\)) alcohols respectively. The 1740 cm\(^{-1}\) and 1175 cm\(^{-1}\) bands indicate an ester. A bump in the ester carbonyl stretch at 1715 cm\(^{-1}\) indicates the presence also of some free fatty acid as an impurity. The NMR spectrum clearly indicates \(\alpha\)-monoglycerides also.

To determine the purity of the \(\alpha\)-monoglycerides after separation twice on analytical TLC plates, ester, glycerol, and terminal vicinal diol analyses were carried out.

(i) Ester Analysis

The following reagents were required for the ester determination.

(a) Ferric perchlorate solution (stock): 5 g of Fe(\(\text{ClO}_4\))^\(3-\) \(\cdot\)6H\(_2\)O in
Fig. 2.3-1. Infrared spectrum of free fatty acids derived from milk
Fig. 2.3-2. Infrared spectrum of monoglycerides derived from milk
10 ml of 70% HClO₄ and 10 ml of water; dilute to 100 ml with cold absolute ethanol. Ferric perchlorate solution (dilute): dilute 4 ml of the stock solution with 3 ml of 70% HClO₄ and make up to 100 ml with cold absolute ethanol.

(b) Alkaline hydroxylamine solution (prepared fresh): mix equal volumes of 4% ethanolic (95%) NH₂OH·HCl and 8% ethanolic (95%) NaOH; centrifuge and use clear supernatant.

(c) Standard ester solution, 8.15 mg methyl arachidate in 25 ml of chloroform (1μ mole/ml).

Procedure: 1 ml of alkaline hydroxylamine reagent was added to 1 mg of the α-monoglycerides in a glass tube, the tube stoppered, and heated in a water bath at 65°C for 2 min. The tube was cooled for 5 min, 3 ml of dilute ferric perchlorate reagent added, and after 30 min the absorbance of the mauve colour read at 530 mμ against a reagent blank. A linear calibration curve was obtained for 0 to 4μ mole of methyl arachidate run simultaneously (Fig. 2.3-3). The unknown was analysed in duplicate to give a value of 2.38 μmole/mg.

(ii) Glycerol Determination

5 mg of the lipid fraction was saponified by refluxing for 90 min in 10 ml of 95% ethanol + 0.5 ml of 33% aqueous potassium hydroxide; 10 ml of 2.5N hydrochloric acid was added and the fatty acids extracted with petroleum ether (40-60°C). The ethanol-water phase was made up to 25.0 ml with water, filtered, and 1 ml aliquots used for glycerol analysis. A standard solution was prepared of 0.5 μmole A.R. glycerol/ml, and the glycerol analysis carried out as follows.

To a sample (or standard) of glycerol in 2 ml of water, 0.1 ml of 10 NH₂SO₄ and 0.5 ml of 0.1 M NaIO₄ were added and left to stand 5 minutes; 0.5 ml of 10% NaHSO₃ were then added, and to an 0.5 ml aliquot
Fig. 2.3-3. Calibration curves for the analysis of glycerol and esterified fatty acid in glycerides
of the whole solution, 5 ml of chromatropic acid reagent was added (100 mg of 1,8-dihydroxynaphthalene-3,6-disulphonic acid in 10 ml of water and diluted with 45 ml of 24 NH\textsubscript{2}SO\textsubscript{4}). The tubes were stoppered (glass), heated in a boiling water bath for 135 minutes, cooled for 30 min, and the absorbance of the purple colour read at 570 μm against a reagent blank.

A linear calibration curve to 1 μmole of glycerol was obtained (Fig. 2.3-3), and a duplicate determination gave a value of \( \frac{0.46 \times 25}{5} \) μmole (mg; i.e. 2.30 μmole glycerol/mg ester.

(iii) Vicinal-diol Determination

The procedure used was practically the same as for glycerol, except periodic acid was used as the oxidizing agent, and sodium arsenite the reducing agent. The procedure was standardized with 0.1-0.5 μmole of glycerol, and the determination carried out on 200 μg samples. A value of 2.10 μmole of lipid containing vicinal diol/mg was obtained.

The 1:1 correspondence between ester group : glycerol/vicinal diol (i.e. 2.38:2.30/2.10) indicates α-monoglycerides. α-monoolein contains 2.80 mole/mg of ester group, glycerol, or vicinal diol, indicating an 85% purity of the monoglyceride isolated from milk. The remaining 15% is probably mostly free fatty acids, but β-monoglycerides may also be present in traces, although these would have been mostly isomerized in analytical TLC procedures.

2.3.5. Isolation of lipid classes from fraction II

The sphingolipids were isolated from the phosphatide fraction by mild alkaline methanolysis. \(^{85}\) To ca. 240 mg of the lipid mixture in 40 ml of chloroform, 40 ml of 0.6 N methanolic sodium hydroxide was
added, and the solution left at room temperature for 1 hour. The solution was then rendered slightly acidic by the addition of 36 ml of 0.7 N HCl, and shaken vigorously. After standing for several hours the chloroform phase was removed, diluted with benzene, and the solvents removed in vacuo. The lipid residue was applied to a 1 metre preparative TLC plate, which was developed in a solvent of composition chloroform-methanol-water (62:25:4). The alkali-stable lipid fractions were eluted from the plate with CHCl₃-CH₃OH (2:1), and the solvents removed in vacuo. Cerebrosides (yield: 12 mg), and ceramide dihexosides (yield: 11 mg), isolated in good yield, gave positive anthrone tests (photograph 2.3-2), and negative phosphate tests (Table 2.3-1). Sphingomyelin, isolated in low yield (45 mg), is indicated by the orange coloured spots shown on photo 2.3-2 (lanes 2 and 3) at $R_F \approx 0.13$. In lane 3 there is observed some anthrone-positive material between the sphingomyelin, and ceramide dihexosides i.e. about the position corresponding to sulphatides (lane 1, $R_F \approx 0.28$); on purification by TLC (removing sphingomyelin and ceramide dihexoside impurities) this fraction gave a still stronger anthrone test, but there was not enough material for characterization. Morrison also found a third anthrone-positive peak in the elution pattern of milk phospholipids, which he suggested may be ceramide trihexoside by noting the similarities between milk glycolipids and blood serum glycolipids. However, sulphatides may also exist, and these compounds could be separated from ceramide oligosaccharides if required.

The cerebrosides and ceramide dihexosides were shown to be glucosyl-ceramides and lactosyl-ceramides respectively, by their behaviour on TLC plates, their infrared spectra, and comparison with some compounds obtained by others from milk and blood serum. On 10% borate-impregnated TLC plates, bovine brain cerebrosides
(galactosyl-ceramides) migrated at a slower rate than the milk cerebrosides \((R_f \sim 0.40-0.55\) for the former, \(R_f 0.52-0.62\) for the latter; solvent \(\text{CHCl}_3-\text{CH}_3\text{OH-H}_2\text{O} (65:25:4)\)). Kean and Young have shown the stronger complexing effect of borate and galactosyl-ceramides compared with glucosyl-ceramides.

The infrared spectra of the glycosides indicate glucosyl-ceramide and lactosyl-ceramide. The spectrum of the ceramide monohexoside shows absorption at 980 cm\(^{-1}\) and 895 cm\(^{-1}\), attributable to \(\beta\)-D-glucosidic bonds; a spectrum of ceramide galactoside (i.e. bovine brain cerebrosides) showed absorption at 880 cm\(^{-1}\) instead of 895 cm\(^{-1}\), which is characteristic of galactosides.

Lactosyl-ceramide was identified by comparing its spectrum with blood serum and buttermilk ceramide dihexosides.

2.3.6. Ceramides from cerebrosides

Of all the sphingolipids containing normal fatty acids or hydroxy-fatty acids, free ceramides give by far the best separation. Thus, the glucosyl-ceramides were converted to free ceramides, not only for this purpose, but also to find any relation between cerebrosides containing either unsaturated, or saturated long-chain bases. The procedure given by Hammarström was used.

10 mg of milk glucosyl-ceramides were dissolved in 0.28 ml of \(\text{CHCl}_3\) and 1 ml of 95% ethanol, 0.6 ml of 0.2 M HIO\(_4\) added and the solution put in a dark place for 3-4 hours. 20 \(\mu\)l of ethylene glycol were then added to destroy excess HIO\(_4\), and after a further 15 min 1.6 ml of water was added. The mixture was centrifuged and the chloroform layer removed. The upper phase was extracted twice with 0.5 ml of portions of \(\text{CHCl}_3\), and the combined extracts washed with
0.4 ml of water, followed by 0.4 ml of water-methanol (1:1). The residue was dissolved in 0.52 ml of CHCl₃, 2.25 ml of 95% ethanol, 0.8 ml of water, and 20 μl of 30% (w/v) aqueous NaOH. Sodium borohydride, 1 mg, dissolved in 0.4 ml of 0.1 N NaOH, was added dropwise, and the solution stirred for 6 hours at room temperature in a loosely stoppered tube. The reduction was stopped by the careful addition of 0.4 ml of 6 N HCl (final concentration is now 0.5 N HCl), and the solution left for 24 hours to bring about complete hydrolysis of the 'polyol'. Water (2 ml) was added, the lower phase removed, and the upper phase extracted with 5 x 2 ml of CHCl₃-CH₃OH (1:1). The combined chloroform extracts were washed twice with water, evaporated to dryness in vacuo, and redissolved in 2 ml of CHCl₃.

The crude preparation, illustrated by TLC on photograph 2.3-3 (lane 2) was purified on a 40 cm analytical TLC plate impregnated with 10% Na₂B₄O₇. Fractions corresponding to saturated dihydroxy bases - normal fatty acids, unsaturated dihydroxy bases - normal fatty acids, and total hydroxy-fatty acid ceramides were isolated. The ratio of normal fatty acid ceramides to hydroxy fatty acid ceramides was about 8:1, and the total yield of pure ceramides was about 5.5 mg (70%).

2.3.7. Ceramides from sphingomyelin

Ceramides were isolated from milk sphingomyelin by enzymatic hydrolysis with phospholipase C.³⁶,⁶⁸,⁸⁵ To 10 mg of sphingomyelin in 3 ml of diethyl ether, 3 ml of 0.1 M Tris buffer containing 0.03 M CaCl₂ and 3 mg (10 units) of phospholipase C (Mann Research Laboratories) were added. The mixture was shaken for 3 hours, after which 6 ml of ether was added and the ceramides extracted. The ether phase was removed, and the aqueous phase extracted with 2 x 6 ml of ether. The
Thin-layer chromatogram of ceramides derived from cerebrosides (1: brain; 2: milk) and sphingomyelin (3: milk; 4: brain).

Solvent: chloroform-methanol (92:8).
combined extracts were evaporated to dryness to produce 7-8 mg of free ceramide (i.e. practically quantitative yield); the crude preparation is illustrated by TLC on photograph 2.3-3 (lane 3), compared to ceramides derived from bovine brain sphingomyelin (lane 4) by the same method. A relatively high concentration of saturated dihydroxy base - normal fatty acid ceramides is observed, as well as the presence of a minute amount of hydroxy-fatty acid ceramides (absent in brain sphingomyelin). The latter are rare in sphingomyelins of animal extracts, but Morrison\textsuperscript{90} has isolated less than 1\% in the sphingomyelin of buttermilk powder. Karlsson et al.,\textsuperscript{71} and more recently, Breimer\textsuperscript{91} have analysed hydroxy-fatty acids in the sphingomyelins of bovine rennet stomach.

2.3.8. \textbf{Fatty acid compositions of the sphingolipids by GLC}

Sphingolipids (ceramides from sphingomyelin or cerebrosides, and lactosyl-ceramides) containing normal or 2-hydroxy fatty acids were hydrolysed by heating the lipid samples (5-10 mg) in 5 ml of 2 N methanolic-HCl in teflon-lined, screw-capped tubes for 5-6 hours at 75-80\degree C. The fatty acid methyl esters were extracted with 4 x 5 ml portions of petroleum ether 40-60\degree, and the solvent removed \textit{in vacuo}. To separate hydroxy-fatty acid methyl esters from normal fatty acid methyl esters in the case of the lactosyl-ceramide, the petroleum ether extract was applied to a 20 cm analytical TLC plate, and the plate developed with ether-hexane (4:6 by vol) to 10 cm height, followed by development with benzene to the top of the plate.\textsuperscript{90} The esters were located with water, and extracted from the silica gel with diethyl ether. Hydroxy-fatty acids comprised about 6\% of the mixture.

Hydroxy-fatty acid methyl esters were converted to the trimethyl-
silyl-ethers by treatment with BSTFA-TMCS (9:1).

Analyses of the fatty acid methyl esters and trimethylsilyl ethers were carried out on 3% SE-30 columns as described in 1.2. Results are tabulated (Table 2.3-2), and example chromatograms are shown for sphingomyelin normal and hydroxy-fatty acid methyl esters (Figs. 2.3-4, 2.3-5).

The compositions of the normal and 2-hydroxy-fatty acids from the three sphingolipids are similar, the principal ones being 23:0 (or h23:0), 24:0 (or h24:0), 22:0 (or h22:0) and 16:0 (or h16:0), 24:1 (or h24:1) and 18:0 (or h18:0) are important to a lesser degree. Lactosyl-ceramide contains larger amounts of the shorter chain, even carbon number fatty acids (12:0 to 18:0) in both its normal and hydroxy-fatty acids.

The compositions of all three sphingolipids are largely similar to those obtained by Morrison for buttermilk powder. However a major difference lies in the relative distribution of hydroxy-fatty acids and normal fatty acids. Morrison found for each sphingolipid hydroxy-fatty acids comprised less than 1% of the total fatty acids. This was found true only for sphingomyelin from Friesan cows; glucosyl-ceramide contained about 12% hydroxy-fatty acids while lactosyl-ceramide contained about 6% hydroxy-fatty acids. These results can be judged by photos 2.3-2 and 2.3-3.

For sphingomyelin and glucosyl-ceramide data is given to determine the relative distribution of normal fatty acids between the saturated and unsaturated long-chain bases. The similarity in fatty acid composition between each species of sphingolipid is apparent, as was also observed by Morrison for sphingomyelin. Thus in milk the biosynthetic pathway of the long-chain bases probably is the same as in other tissue i.e. they are synthesised in vivo from saturated
<p>| Fatty acid | Sphingomyelin | | Glycosyl-ceramide | | Lactosyl-ceramide |
|-----------|---------------|----------------|------------------|------------------|
|           | Normal I*     | Normal II*     | Hydroxy          | Normal I*        | Normal II*       | Hydroxy          | Normal | Hydroxy |
| 12:0      | 0.1           | tr             | 0.1              | -                | -                | -                | 1.1    | tr      |
| 14:0      | 0.7           | 0.4            | 1.9              | 1.2              | 0.3              | 1.9              | 5.9    | 1.4     |
| 15:0      | 0.2           | 0.2            | 0.2              | 0.1              | 0.1              | tr               | 0.4    | 0.1     |
| 16:0      | 14.0          | 15.8           | 8.3              | 9.8              | 10.8             | 9.7              | 16.6   | 13.5    |
| 16:1      | 0.2           | 0.1            | -                | 0.3              | 0.1              | -                | 0.5    | -       |
| 17:0      | 0.3           | 0.3            | -                | 0.3              | 0.2              | 0.5              | 0.4    | 0.8     |
| 18:0      | 2.9           | 2.2            | 3.9              | 2.8              | 1.3              | 3.8              | 10.4   | 4.2     |
| 18:1      | 0.2           | 0.2            | 0.2              | -                | -                | -                | 0.5    | 0.1     |
| 18:2      | tr            | 0.1            | -                | -                | -                | -                | -      | -       |
| 19:0      | 0.8           | 0.4            | 0.6              | 0.4              | 0.2              | tr               | 0.1    | -       |
| 20:0      | 1.1           | 0.7            | 1.6              | 1.8              | 0.7              | 2.3              | 1.1    | 1.2     |
| 20:1      | 0.6           | 0.3            | -                | 0.1              | 0.1              | 0.1              | 1.1    | -       |
| 21:0      | 1.4           | 0.8            | 1.5              | 2.1              | 0.2              | 1.0              | 1.2    | 1.3     |
| 21:1      | 0.2           | tr             | -                | 1.9              | 0.8              | -                | -      | -       |
| 22:0      | 17.4          | 18.6           | 16.5             | 22.3             | 23.9             | 18.9             | 17.2   | 16.2    |
| 22:1      | 2.0           | 0.3            | -                | 2.1              | 1.0              | -                | -      | -       |
| 23:0      | 23.4          | 24.3           | 32.0             | 25.9             | 29.8             | 34.7             | 19.5   | 24.8    |</p>
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| I and II indicate the source of the fatty acid. I: saturated. LCB-normal FA ceramides; II: unsaturated LCB-normal FA ceramides.
Fig. 2.3-4. Gas-liquid chromatogram of normal fatty acid methyl esters derived from bovine milk sphingomyelin. Column: 3% SE-30 cm 100-120 mesh Gas Chrom Q; temp. programming 160°C to 310°C at 5°C/min; N₂ flow rate, 20 ml/min.
Fig. 2.3-5. Gas-liquid chromatogram of hydroxy-fatty acid methyl esters derived from bovine milk sphingomyelin. Column: 3% SE-30 cm 100-120 mesh Gas Chrom Q; temp. programming 160°C to 310°C at 5°C/min; N₂ flow rate, 20 ml/min.
fatty acids, and the trans double bond is formed by desaturation of the saturated bases.92

2.3.9. Discussion

Some differences have been pointed out in the sphingolipids of Fresian cows compared to buttermilk which has been obtained from a variety of breeds. The dietary significance of the sphingolipids is a subject about which little is known i.e. how sphingolipids are absorbed and metabolised from foodstuffs. Milk is one of the most important foods consumed, and such data as presented here will become important in this aspect at some future date. Long-chain bases need also to be analysed for the sphingolipids to see what similarities exist. If they are similar in composition then certain conclusions could be drawn e.g. the possibility of a common ceramide pool as an intermediate in the synthesis of sphingolipids.

Free ceramide was in too low a concentration to be isolated here, but Fujino77 has isolated a small quantity of the lipid from Holstein cow milk. Its composition proved to be similar to the composition of milk sphingomyelin isolated by Fujino et. al.95 Mori et. al.97 and to the sphingolipids isolated by Morrison36,90 from buttermilk powder. This indicates that free ceramide may be the precursor of the complex sphingolipids, as it has already been definitely shown that sphingomyelin is derived from ceramide.43 At the same time, however, the breakdown of sphingolipids to ceramide by enzymatic hydrolysis probably occurs in living tissue, as suggested by Gatt.96 In the case of milk, the irreversible conversion of sphingomyelin to ceramide, by the action of sphingomyelinase present in the milk, has been demonstrated (author's M.Sc. Qual. thesis, 1973).

Of interest would be the analysis of the polar lipids of goat
milk, to ascertain what similarities exist with cow milk, and possibly how diet and breed affect the lipid composition. This animal has been untouched in this field to my knowledge, and at present some work is in progress on milk from a Saanen goat.

2.4. **Analysis of Serum Lipids by Spectrodensitometry**

Blood sera from 68 normal, healthy male adults from the EZ Company were analysed for cholesterol, free ceramide and monoglyceride by spectrodensitometry.

2.4.1. **Extraction and TLC**

Sera (0.5 ml) were treated with chloroform methanol 2:1 (10 ml) for 30 minutes, and the solution partitioned with 2 ml of 1% aqueous sodium chloride. The chloroform layers were removed, filtered through Whatman 1 PS filtered papers, and the chloroform removed under vacuo. The lipid residues were each taken up in 200 μl of chloroform; 10 μl of each of the 68 samples were applied to TLC plates and analysed as described in 1.4. A typical TLC plate illustrating the lipids extracted from seven serum samples (photograph 2.4-1) shows clearly triglycerides at the solvent front, cholesterol at Rf 0.5, and phospholipids at the origin. The white strip just above the origin is a Na₂CO₃ band, which acts as a barrier for the free fatty acids. Monoglycerides plus OHFA ceramides, and non-OHFA ceramides, occur as minor constituents at Rf values of approximately 0.11 and 0.17 respectively (other lipids could be present in this area, also).

2.4.2. **Results**

The results of the serum analysis are given in Table 2.4, and
were obtained by direct comparison of peak heights with the standard curves of Fig. 1.4. The mean values of free cholesterol (67.3 ± 24.2) and total cholesterol (241 ± 42.2) compare well to the means obtained by Chedid et. al.33 for 70 apparently healthy adults (53 ± 12.2 mg % and 217 ± 27.8 mg % respectively). The ratio of esterified cholesterol to free cholesterol averages at 2.6.

The monoglyceride mean (15.8 ± 10.5 mg %) is high compared to Chedid's result (<5 mg %). OHFA ceramides are not usually present in healthy serum, which indicates the possibility of other types of lipids which migrate to the same area on TLC plates as monoglycerides; in actual fact, 4-5 minor bands were observed in this area. The analysis of the combined serum monoglycerides is given in 2.4-3 by a different method; a value of 5 mg % was obtained.

The control serum in Table 2.4 showed a value of 63 mg % for free cholesterol. To determine the amount of total cholesterol by spectrodensitometry, the serum (0.5 ml) was first saponified with 5 ml of 0.3 N methanolic NaOH for 2 hours at 80°C in a teflon-lined screw-capped tube. The serum lipids were then extracted 4 times with petroleum ether (40-60), 5 ml each time, and analysed for total cholesterol. A value of 217 mg % was obtained, which is about the expected value for a healthy adult.

The results obtained for cholesterol above are of the right order, but there are some factors which may contribute to inaccuracy. The method used for extraction1 does not exclude the possibility of some retention of cholesterol in the methanolic-aqueous layer. In actual fact a little free cholesterol (ca. 5%) was found left in this layer on exhaustive extraction with CHCl₃-CH₃OH mixtures. The possibility of cholesterol-protein complexes was checked by boiling for 10 min a dilute serum sample (½ ml serum to 3 ml with water) and
TABLE 2.4
Survey of EZ Workers

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<td>Mean ± ISD</td>
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(a) Results for total cholesterol obtained by Dr. R.S. Parsons (Hobart), by the Liebermann-Burchard reaction.
(b) Blood serum taken from myself; results averaged from a number of individual values.
analysing for cholesterol in the normal way. Results were the same as for normal extracted serum. The presence of other types of lipids in a mixture may affect the separation or distribution of the TLC spot. A 'synthetic serum' was prepared of composition tripalmitin-cholesterol-ceramide-phospholipids (80:20:1.4), and compared to free cholesterol and normal serum cholesterol analyses. The results were identical.

2.4.3. Analysis of serum monoglycerides using $\text{IO}_4^-$--Schiff's reagents

300 mg of total lipids from 30 ml of pooled serum were treated with $\text{CH}_2\text{N}_2$ in methanolic ether, and the residue dissolved in 10 ml of $\text{CHCl}_3$. 30 μl (900 μg lipid) of this solution was applied to a Merck prepared plate as described in 1.4.2. The other six lanes on the same plate were taken up with monoglycerides obtained from milk lipids. The plate was developed in $\text{CHCl}_3-\text{CH}_3\text{OH}$ (93:7), dried, and immersed slowly and evenly into a 1% solution of $\text{NaIO}_4$. After half minute excess reagent was quickly removed by blotting with filter paper, and the plate placed in a tank saturated with $\text{SO}_2$ for 15 minutes. It was then sprayed lightly and evenly with Schiff's reagent, and placed back into the $\text{SO}_2$ tank for 1 hour. The pink p-rosaniline spots were scanned immediately after removal from the $\text{SO}_2$ atmosphere; the result obtained was 4.5 μg of monoglyceride/900 μg of total lipid, or about 5 mg %.

The accuracy of any spectrodensitometric method is largely based on the stability of the colour produced on the plates. This reaction has not been considered for densitometry before, due to the relative instability of the pink-coloured spots (they do not seem to visibly deteriorate on standing for some hours, however, and the time for scanning is less than 1 minute). The result, 5 mg %, is a typical
value for normal serum.

An attempt was made to remove monoglycerides before application of the lipid mixture to the plate, by mild alkaline methanolysis. 2 mls of 0.6 N methanolic NaOH was added to 10 mg of serum lipids in 2 mls of CHCl₃. The solution was left to stand at room temperature for 18 hours, after which time it was noticed that free ceramides, also subjected to the same treatment, began to degrade. TLC of the serum lipids, on a plate containing a strong Na₂CO₃ barrier, showed the existence still of monoglycerides.

2.4.4. Analysis of serum ceramides using t-butylhypochlorite-KI/starch

Ceramides are usually present in minute quantities in lipid extracts from normal animal tissue. Best results were obtained using a ceramide-rich fraction rather than total lipids. 300 mg of total serum lipids were applied to 20 cm x 2½ cm column of activated silicic acid saturated with CHCl₃. A fraction collector was used, and with a flow rate of about 1 ml/minute the lipids were eluted as follows (TLC monitored): neutral lipids, including most of the cholesterol, 80 mls of CHCl₃; ceramide-rich fraction (also containing a little cholesterol, cerebrosides, FFA, etc.), about 200 ml of CHCl₃-CH₃OH (4:1). The crude ceramide fraction (~ 4 mg) was treated with dilute methanolic NaOH, followed by CH₂N₂ to esterify FFA. The residue was dissolved in 0.5 ml of CHCl₃, and 40 µl applied onto a silica gel plate prepared in the laboratory (TLC plates purchased from Merck were found unsuitable for this method). For standards, non-OHFA ceramides, ex-bovine brain, were used. The plate was developed as described in 1.4.2, dried, and sprayed evenly with a 2% solution of t-butylhypochlorite in cyclohexane. After drying under a cold air blast for
1 hr, the plate was then sprayed lightly with a 1% solution of KI-starch, and the blue-black spots scanned immediately. The plate is illustrated by photograph 2.4-2 and the scan given by Fig. 2.4. Values based on serum, are about 1 mg % non-OHFA ceramides, and 0.04 mg % OHFA ceramides. These results are probably not as reliable as those obtained for monoglycerides by the I$_4$/Schiff's method, as the plates darken quickly (within 10 minutes) on exposure to the atmosphere, and I$_2$ is lost from the spots. However the standards are subject to the same conditions, and comparable results should be obtainable if no time is wasted in analysis.
TLC plate illustrating blood serum lipids, detected with phosphomolybdic acid.

TLC plate illustrating method of analysis for ceramides Detection: t-butyl hypochlorite then KI-starch. Spots appear blue-black for lipids containing NH group.
Fig. 2.4. Quantitation of ceramides by densitometry. A = non-OHFA ceramide, and B = OHFA ceramide, from 25 mg normal blood serum lipid.
3.1. Side-reactions in the synthesis of ceramides using carbodiimides

Ceramides are conveniently prepared in 60-70% yield by direct coupling of long-chain base and fatty acid in the presence of the mixed carbodiimide, N-ethyl-N'-dimethylaminopropylcarbodiimide. By-product formation is extensive as many functional groups are exposed, but the major by-products were shown to be N-acyl ureas, and to a lesser extent, fatty acid methyl ester.

An example of crude ceramide preparation (n-nervonoyl-D,L-erythrosphingosine) is illustrated, by TLC, on photograph 3.1-1. The synthetic "non-hydroxy fatty acid" ceramide occurs on this plate at $R_f \approx 0.65$, while the spots at $R_f \approx 0.43$ and $R_f \approx 0.21$ proved to be N-acylureas, by analysis of their infrared and mass spectra. The product near the solvent front ($R_f \approx 0.9$) was shown to be principally the methyl ester of the fatty acid used in the ceramide preparation.

3.1.1. Preparation of ceramides

The following ceramides were prepared (about 5 mg of each): 18:1-d18:0, 18:2-d18:0, 20:0-d18:0, 22:0-d18:0, and 18:0-d18:1. 5 mg of long-chain base (i.e. about 17 µ moles of dihydrosphingosine or sphingosine) and 12-15 mg of fatty acid (about 40 µ moles) were dissolved in 2.5 ml of $\mathrm{CH}_2\mathrm{Cl}_2$, 0.5 ml of $\mathrm{CH}_3\mathrm{OH}$ and 2.5 ml of $\mathrm{CH}_3\mathrm{CN}$. 1.5 ml of a 10% solution of the carbodiimide in $\mathrm{CH}_2\mathrm{Cl}_2$ was added, and the solution heated overnight at 45°C in a teflon-lined screw capped tube. The solution was then transferred to a separatory funnel with 50 ml of diethyl ether (distilled), and the mixture was washed three times with 15 ml of 5% aqueous $\mathrm{NaHCO}_3$, followed by three
1: Non-OHFA ceramides; 2: Synthetic N-nervonoyl-DL-erythro-trans-4-sphingenine (unpurified).
washings with 15% NaCl solution, and then twice with water. A little ethanol was added, and the solvent removed in vacuo. Each ceramide preparation was applied to a 40 cm x 20 cm analytical TLC plate, and the fractions corresponding to ceramide and N-acylurea eluted from the silica gel after development.

3.1.2. Analysis of infrared spectra

The IR spectrum of typical ceramides (Fig. 3.1-1, trans- 18:1-d18:1, Fig. 3.1-2, 22:0-d18:0), in CC14, shows the OH and NH stretching vibration between 3600 and 3100 cm\(^{-1}\), the C=O stretch (amide I band) at 1650-1630 cm\(^{-1}\), and NH band plus some C-N stretch (amide II band) at 1550-1530 cm\(^{-1}\). For ceramides containing sphingenine, a sharp absorption peak occurs at 970-960 cm\(^{-1}\), absent in sphinganine and phytosphinganine ceramides. This peak is attributable to the trans-double bond C-H stretch; for the ceramide trans- 18:1-d18:1 it is especially strong as the fatty acid is also trans- unsaturated. Ceramides have no ester carbonyl absorption (\(\nu\)1740 cm\(^{-1}\)).

The spectra of the proposed acylureas (e.g. Figs. 3.1-3, 3.1-4) were practically superimposable for each ceramide preparation, and for different ceramide preparations the difference lay only in the nature of the fatty acid component. Major bands occur at 3,270 cm\(^{-1}\) (N-H stretch), 1705 cm\(^{-1}\) and 1655 cm\(^{-1}\) (C=O stretching frequencies for urea and tertiary amide), and 1525 cm\(^{-1}\) (NH band). Also very characteristic of the gem-dimethylamino group in the triplet which occurs between 2800 and 2720 cm\(^{-1}\); these bands are observed at 2800 cm\(^{-1}\), 2770 cm\(^{-1}\) and 2750 cm\(^{-1}\). The C-N stretching frequencies occur between 1230 cm\(^{-1}\) and 1100 cm\(^{-1}\).
Fig. 3.1-1. Infrared spectrum of N-elaidoyl-D,L-erythrosphing-4,t-enine (trans 18:1-d18:1) in CC1₄
Fig. 3.2-2. Infrared spectrum of N-behenoyl-D,L-racemic-dihydrosphingosine (22:0-d18:0) in CCl₄. The band at 1600 cm⁻¹ may be due to the C=O stretch for the threo- isomers, although the individual spectra of the pure D,L-erythro and D,L-threo isomers show only one amide I band (1650-1630 cm⁻¹). This is being further investigated.
Figs. 3.2-3 (A) and 3.2-4 (B). Infrared spectra of N and N'-behenoyl-N-ethyl-N'-(dimethylaminopropyl)carbodiimides. A represents the less mobile compound, while B represents the more mobile compound on TLC plates.
3.1.3. **Analysis of mass spectra**

The mass spectra of synthetic ceramides is described elsewhere.\(^\text{102}\)

The molecular weight of each of the proposed acylureas is indicated by molecular ion peaks in their mass spectra e.g. for N-ethyl-N'-((dimethylaminopropyl)-N (or N')-behenoylurea (Figs. 3.1-5 and 3.1-6), \(M^+\) occurs at m/e 495; for the oleoylurea, \(M^+\) occurs at 437. Ions common to the mass spectra of all the acylureas are m/e 200 for \(C_2H_5-N-(CH_2)_3-N(CH_3)_2\), and m/e 57 for \(CH_2=N(CH_3)_3\).

Since there are two acylurea fractions for each ceramide preparation it could be assumed that the difference lies in which nitrogen the acyl group is bonded to. Khorana\(^99,100,101\) suggests the following mechanism for the formation of acylureas from carbodiimides and carboxyl acids.

\[
\text{RN=C=NR} + \text{R'COOH} \rightarrow \text{RN=C=NHR} \rightarrow \text{RN=CNHR} \]

The carbodiimide is protonated, followed by attack of the acid anion to form an intermediate which rearranges to the acylurea (I).

Alternatively, a second proton may overtake the O to N migration,
Fig. 3.1.5. Partial mass spectrum of N-benoyl-N-ethyl-N'- (dimethylaminopropyl)carbodiimide.
Fig. 3.1-6. Partial mass spectrum of N'-behenoyl-N'-(dimethylaminopropyl)-N-ethylcarbodiimide
and a urea and acid anhydride (II) forms as the end product, via the intermediate cation as shown.

In unsymmetrical carbodiimides, the initial attachment of the proton would be expected to occur preferably to the nitrogen atom with higher electron density. The acyl group would thus attack the less basic nitrogen. With N-ethyl-N₁-(dimethylaminopropyl)-carbodiimide the acylurea most probably formed is

\[
\begin{align*}
\text{C}_2\text{H}_5 & - \text{N} - \text{C} - \text{NH(CH}_2\text{)}_3\text{N(CH}_3\text{)}_2', \\
\text{C}=\text{O} & \\
\text{R} &
\end{align*}
\]

as the dimethylaminopropyl group would have a stronger inductive effect than the ethyl group.

3.2. **Structural problems**

Future interests in work carried out will lie in the chemistry of the formation of cyclic compounds, in particular oxazolines, of natural ceramides, (and sphingolipids in general), ceramide analogues, and related compounds. The following process seems chemically possible.

It seems that this aspect has not been studied in the sphingolipid field. Certain mechanisms have been postulated (Welsh\textsuperscript{38}) involving oxazoline intermediates, to explain the stereochemical course of acyl migrations in derivatives of ephedrine and \(\psi\) ephedrine. These are as follows.
The formation of threo-sphingosines as well as natural erythro-
sphingosines on acidic methanolysis of sphingolipids\textsuperscript{103,58} has been 
explained by the inversion mechanism. This point can be taken up in 
more detail, since it is possible that the stable oxazoline (I) is in 
equilibrium with the rearranged product (II).

Also relevant to the chemistry of animal sphingolipids is the 
possible formation of trans-epoxides between the C4-C5 carbons of the 
sphingosine moiety. Hydrolysis would lead to cyclic structures of 
the tetrahydrofuran type, which have been isolated from natural 
products containing phytosphingosine by acidic methanolysis, and in 
the free state in yeast cerebrins.\textsuperscript{104}
REFERENCES


