A STUDY OF CERAMIDES AND RELATED COMPOUNDS

by

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Submitted in fulfilment of the requirements

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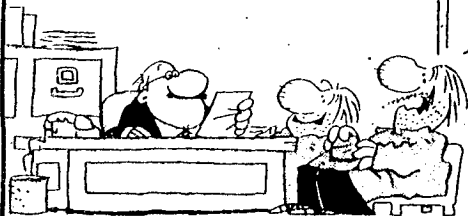UNIVERSITY OF TASMANIA

HOBART

OCTOBER, 1978

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 institution and contains no copy or paraphrase of material previously published or written by another person, except when due reference is made in the text.

E. George

ACKNOWLEDGEMENTS

Abstract

1. The thesis describes the synthesis of new compounds including
    both N- and O-acyl sphingoids, N- and O-acyl β-hydroxyaminoacids,
    related oxazolines, and aminoacid esters and amides of lipids.

2. Some minor lipids chromatographed with ceramides from commercial
    and fresh biological sources were identified as partially oxidised
    cerebrosides and monoglycerides respectively.

3. Chromatographic methods (TLC and GLC) were elaborated for the
    study of sphingolipids with special reference to the study of

    > a) acyl migration;
    > b) ageing;
    > c) stereochemistry.

4. Resolution of groups of erythro and threo sphingoids by TLC and
    separation of individual diastereoisomers by GLC permitted the
    assignment of erythro configuration to natural sphingoids of brain.

5. It was shown that synthetic oxazolines derived from sphingoids and
    containing O^1 and O^3 in the ring were predominantly of the former
    kind if derived from erythro sphingoids, and of the latter kind if
    derived from threo sphingoids.

6. All oxazolines, except those derived from erythro sphingenine and
    containing O^3 in the ring, underwent acid hydrolysis without change
    of configuration.

7. Synthetic O-acyl sphingoids readily changed to ceramides in neutral
    or alkaline media but the conversion of ceramides to O-acyl isomers
    could not be brought about by acid; oxazoline intermediates were
    not found.

8. Neither oxazolines nor O-acyl sphingoids have been found in fresh or
    aged ceramide solutions. Changes of ceramides on ageing are
    ascribed to photo-oxidation.

9. The fatty acid composition of sphingolipids from the milk of Friesian
    cows was surveyed.

10. The neutral lipids of 68 workers exposed to lead were assayed.

11. Studies of aminoacids in neutral lipid fractions suggest the presence
    of some covalently bound aminoacid-lipid combinations.

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INTRODUCTION

1. Definition

This work is a report of analytical, synthetic, and mechanistic studies of ceramides and some of their constitutional or chromatographic analogues.

2. Nomenclature, abbreviations, symbols and other conventions

(a) Nomenclature

The recently revised nomenclature for sphingolipids[1], will be adopted where possible.

The term "sphingoids" will refer to 2-amino-1,3-octadecanediol, its homologues and stereoisomers, and to the hydroxy, methoxy, and unsaturated derivatives of these compounds. "Sphingenines" and "sphinganines" denote the stereoisomers of 2-amino-trans-4-octadecene-1,3-diol and 2-amino-1,3-octadecanediol, respectively.

The configuration of substituents on sphingoids or 2-hydroxy fatty acids is specified where necessary by the prefixes "D" or "L" (not "R" or "S") which follow the locant of substitution. These prefixes specify the orientation of the functional groups to the right or left, respectively, of the carbon chain when written vertically in a Fischer projection with C1 at the top. The notations "e" (erythro) or "t" (threo) are used when two adjacent substituents have the same or opposite configuration, respectively. The term "rac" (racemic) represents a mixture of the DLe and DLt-stereoisomers. Examples of sphingoids differing in configuration are given (Fig. 1).
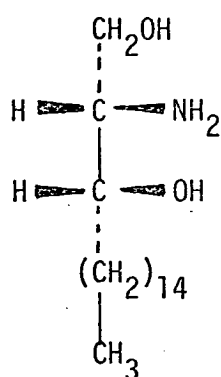
2D,3D-sphinganine, or
De-sphinganine
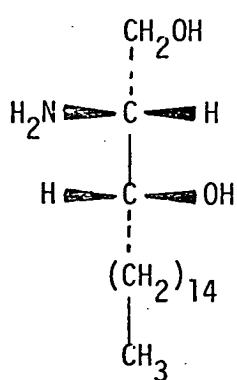
2L,3D-sphinganine, or
Dt-sphinganine
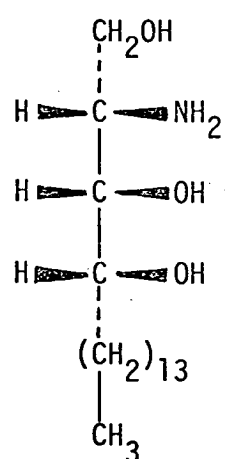
4D-hydroxy-De-
sphinganine

Fig. 1
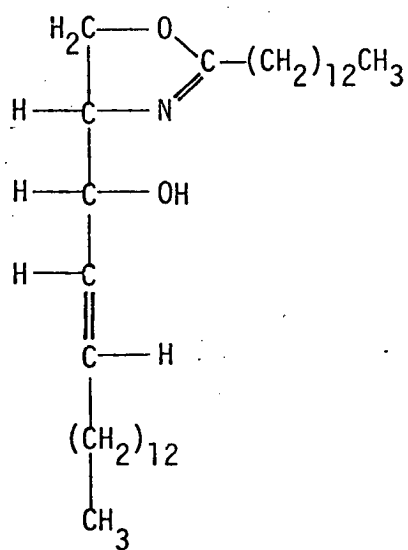
2-tridecyl-4-(1D-hydroxy-trans-2-hexadecenyl)-2-oxazoline

Fig. 2

The terms "ceramide" and "N-acyl sphingoid" are equivalent.

The generic terms, sphingolipids, ceramides, sphingomyelins, and glycosphingolipids, will be used. The last-mentioned lipids include all compounds containing at least one monosaccharide, such as glucosylceramides (or generally, cerebrosides), lactosylceramides, psychosines, sulphatides, and gangliosides. The configuration of the sphingoids in these groups of compounds, generally believed to be De, will be specified where known, e.g. DLt-sphingomyelin.

Acyl sphingoids include N-acyl, O-acyl (e.g. 3-O-palmitoyl sphingenine), and di- or tri-acyl compounds in which the locations of the acyl groups are indicated so as to distinguish 1-O,3-O, 1-O,N, and 3-O,N derivatives. The term "ceramide esters" implies that one of the hydroxyls of ceramides is esterified, as in the known cerebroside esters of brain.

2-Oxazolines derived from sphingoids shall be termed "sphingoid-oxazolines". The oxygen not participating in the ring is indicated as a hydroxyl kane or hydroxyalkene substituent, e.g. 2-tridecyl-4-(1D-hydroxy-trans-2-hexadecenyl)-2-oxazoline (Fig. 2).

(b) Abbreviations

In addition to the common abbreviations such as Me (methyl) and Trid (tridecyl), others will be used, e.g. 1h-Hexad$^{\Delta 2}$, for 1-hydroxy-trans-2-hexadecenyl.
hMe, for hydroxymethyl.
cEy, for carboethoxy.

TLC and GLC stand for thin-layer chromatography and gas-liquid chromatography, respectively.

IR, UV, NMR, and MS spectra refer to infrared, ultraviolet, nuclear magnetic resonance, and mass spectroscopy, respectively.

Trimethylsilyl and dinitrophenyl are abbreviated as TMSi and DNP, respectively.

NFA, for non-hydroxy fatty acid, and HFA, for 2-hydroxy fatty acid, will be used for the classification of sphingolipids.

(c) Symbols

Non-hydroxy fatty acids (or methyl esters) are symbolised by carbon number:number and position of double bonds; the double bond has the cis arrangement unless otherwise indicated. Thus, 18:1(9) stands for oleic acid. In the case of 2-hydroxy fatty acids, the configuration of the hydroxy group is specified, and the letter h precedes the symbols. Thus, D-h18:0 stands for D-2-hydroxystearic acid.

Sphingoids are symbolised by carbon number:number of double bonds, but distinguished from fatty acids with the letters d or t, which stand for dihydroxy and trihydroxy respectively (i.e. the number of hydroxy groups in the sphingoid). The ones most encountered are d18:1 (sphingenines), d18:0 (sphinganines), and t18:0 (4D-hydroxy-De-sphinganine); the double bond of d18:1 occurs at C4, and has the trans-configuration unless otherwise stated.

The notations used for acyl sphingoids are combinations of the symbols described for fatty acids and sphingoids, e.g. D-h18:0-Dt-d18:1 (a ceramide), and 3-0-18:0-Dt-d18:1 (an 0-acyl sphingoid).

(d) Other conventions

Melting points are shown as °, which refers to °C.

IR spectral frequencies are given in cm^{-1} (wavenumbers); stretching and bending modes are indicated by the symbols ν and δ, respectively.

NMR spectral data are given in τ values.

3. Nature and scope of investigations

Earlier work in this Department[2] drew attention to problems associated with the TLC analysis of ceramides.

The identification and estimation of ceramides by means of TLC is rendered difficult or critically unacceptable through the presence of other lipids of comparable mobility. The main contaminants were identified as cholesterol, fatty acids, and α-monoglycerides. Simple techniques sufficed to separate them from ceramides.

Gradual loss of homogeneity in purified ceramide preparations indicates chemical change. The identification of the reaction products, and the establishment of the mode of their formation from parent ceramides, constitutes a substantial part of the work presented in this thesis. The most likely products arising in ageing ceramide solutions were the 3-0-acyl sphingoids, which could be derived from ceramides by acyl migration through oxazoline (or oxazolidine) intermediates (Fig. 3).

Other products of interest were anhydrides, e.g. tetrahydropyrans (Fig. 4), which could form via an epoxide[3], and 1,4-tetrahydrooxazines (Fig. 5), which may arise from HFA ceramides.

Model compounds of acylated sphingoids and sphingoid-oxazolines were prepared, and their chemistry studied with their presence in natural products in view. The presence of at least two asymmetric centres gave rise to problems of distinguishing between stereoisomers. Related model compounds derived from β-hydroxy amino acids were also prepared and used to study acyl migrations and stereochemical conditions.

ceramides   oxazolines   3-O-acyl sphingoids

1-O-acyl sphingoids
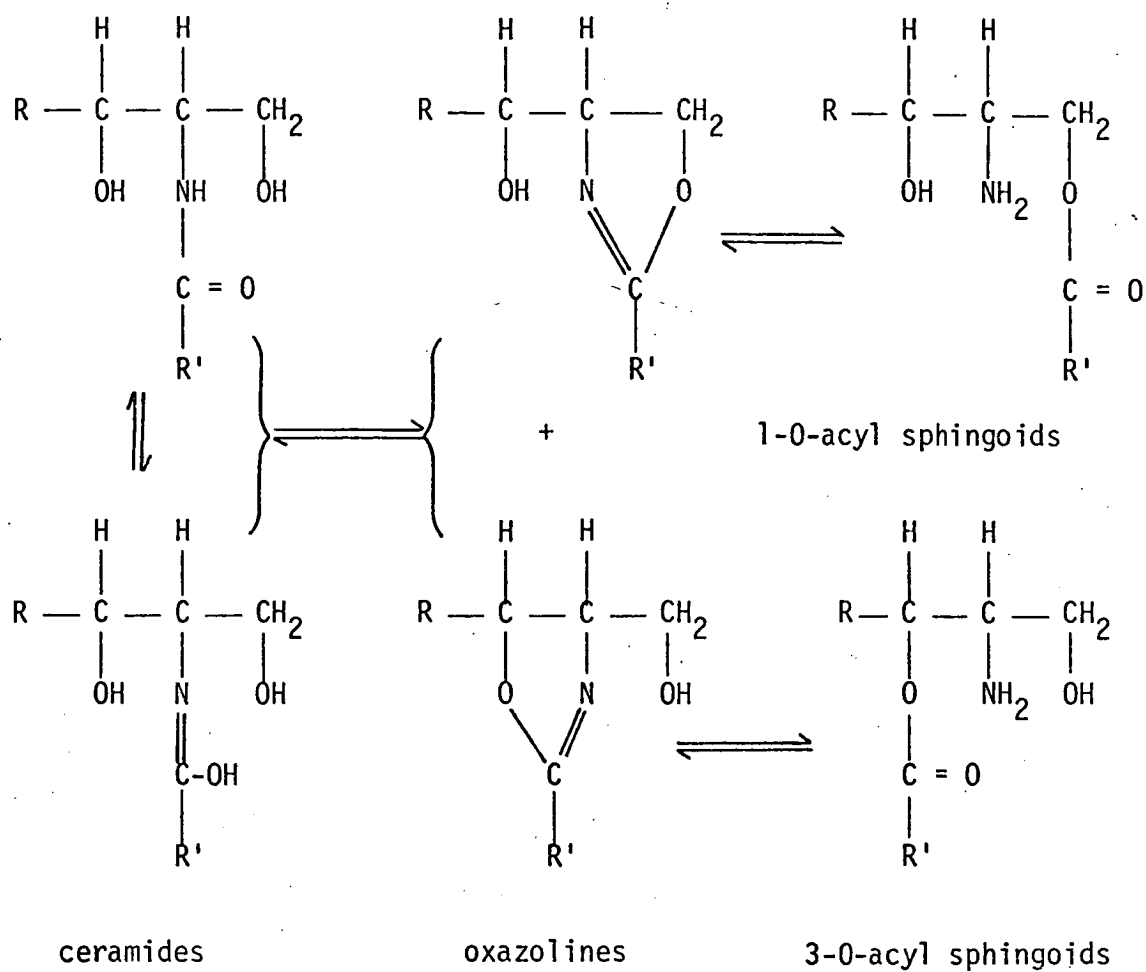
Fig. 3
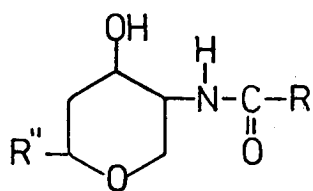
tetrahydropyrans

Fig. 4
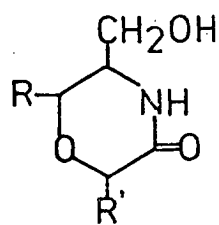
1,4-tetrahydrooxazines

Fig. 5

Legend

R,R', and R" are long-chain alkyl groups.

Some minor problems were met with in connection with the TLC analysis of ceramides.

(i) The finding of amino acids in lipids of comparable mobility to ceramides suggested the presence of lipoamino acids, which might have been derived from glycerol, fatty acids, cholesterol or sphingoids.

(ii) In some commercial ceramide preparations anomalous TLC spots, slower than the parent ceramides, were detected. These were shown to be due to polyols formed in the course of preparation of the ceramides.

(iii) Ceramides and other lipids exposed to subdued sunlight for relatively short periods of time underwent photooxidation, giving rise to anomalous TLC spots.

4. Structure of the thesis

Work submitted in the form of this thesis is divided as follows:

CHAPTER 1. Materials, methods for their isolation, and some qualitative and quantitative analytical procedures.

CHAPTER 2. The analysis of major contaminants in natural and commercial ceramide preparations. Evidence for new naturally occurring minor lipids derived from sphingoids. The synthesis and chemical nature of acyl sphingoids, sphingoid-oxazolines, and related compounds.

CHAPTER 3. The stereochemical analysis of acyl sphingoids and sphingoid-oxazolines.

Some of the methods and findings of Chapters 1 to 3 have found application in short projects reported in the Appendices.

5.   Review

(a) Controversies relating to the existence of natural threo-
sphingolipids

The major sphingoids isolated from tissues are De-d18:1 and
De-d18:0, from animals[4-7], and t18:0, from plants[8].   The biosynthesis
of sphingolipids requires e-sphingoids, but their diastereoisomers can
play an equally active part.   All isomers have been found to accept
the acyl group to form ceramides enzymatically in vitro[9] and in vivo[10].
Psychosine may be prepared from a variety of e- and t-sphingoids,
especially the former[11].

In the biosynthetic pathways leading to sphingomyelin, several
authors were sceptical of the role of t-d18:1 as an active lipid
acceptor.   The N-acylation of sphingenyl-1-phosphorylcholine in rat
brain gave a product which showed the chromatographic properties of
t-sphingomyelins[12].   This finding was questioned, since the
sphingenine synthesised in rat brain was known to have the
e-configuration[6].   Some thought that the sphingenyl-1-phosphoryl-
choline isomerised during its preparation[13], while others noted
discrepancies in its analytical data[14].   Recent claims that sphingenyl-
or sphinganyl-1-phosphorylcholine function neither as intermediates
in the biosynthesis nor in the degradation of sphingomyelin have only
added to confusion[15].

Evidence for natural t-sphingolipids was also obtained from investigations on the biosynthesis of sphingomyelin from CDP-choline and ceramides[16]. Using an enzyme system from chicken liver, t-ceramides were the only active substrates, even though sphingoids from chicken liver have the e-configuration. More recently, another enzyme (cholinephosphotransferase) has been found to catalyse the reaction using either t- or e-ceramides[14].

(b) Acyl migrations in systems related to ceramides

The mechanism and stereochemical course of acyl migrations between nitrogen and oxygen atoms on adjacent carbons have been critically investigated; in many cases oxazoline intermediates were prepared[17-23]. The most important results of such investigations are summarised in Fig. 6. In general, dry hydrogen chloride at room temperature converts cis-acylaminoalcohols (II) to esters (III) with retention of configuration; (III) reverts to (II) on treatment with alkali. The trans-acylaminoalcohols (I) are unaffected by this mild treatment, but heating with hydrogen chloride brings about acyl migration accompanied by an inversion of configuration, and ester (III) is formed.

Retention and inversion mechanisms have been postulated to explain configurational changes that occur during treatment of acylaminoalcohols with acid (Fig. 7)[17]. The possibility of inversion prior to acyl migration has also been considered[19]. The mechanisms are thought to involve transient cyclic hemiacetals (V) rather than oxazolines (VI) (Fig. 8). The O to N migration in N-(2-hydroxyethyl)-benzamide did not yield an oxazoline intermediate, but in the N to O migration, some 2-phenyloxazoline was isolated[24]. This suggests that in similar systems,
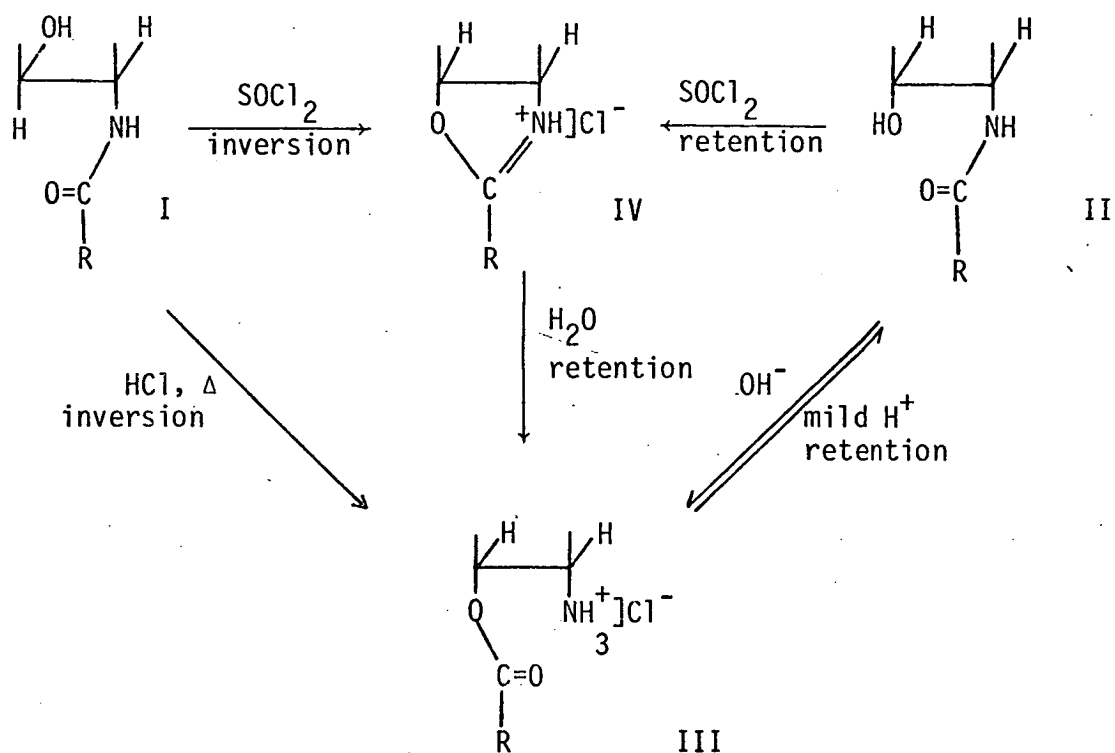
Fig. 6
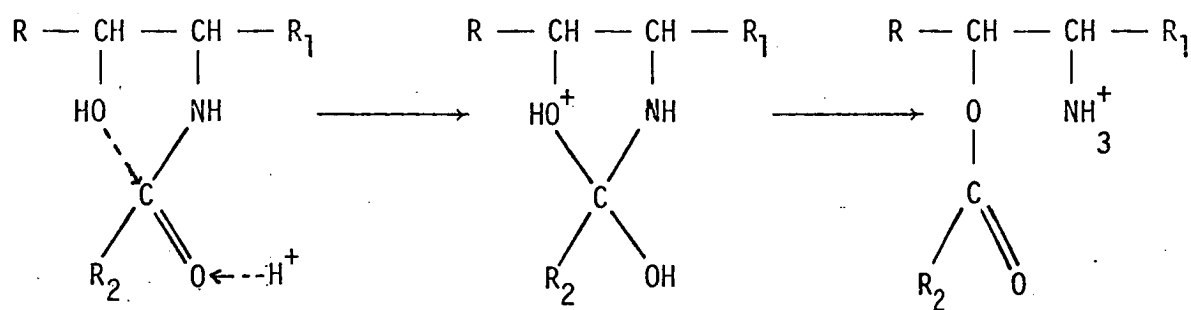
A. Retention mechanism
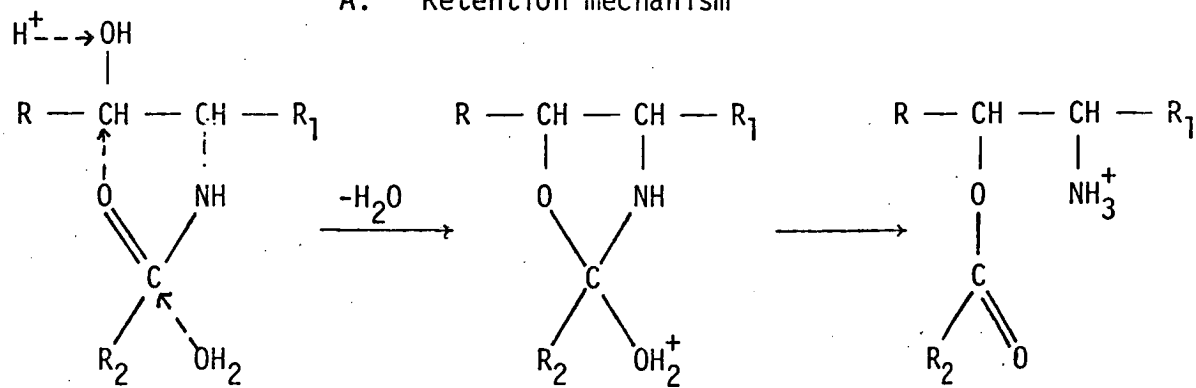
B. Inversion mechanism

Fig. 7
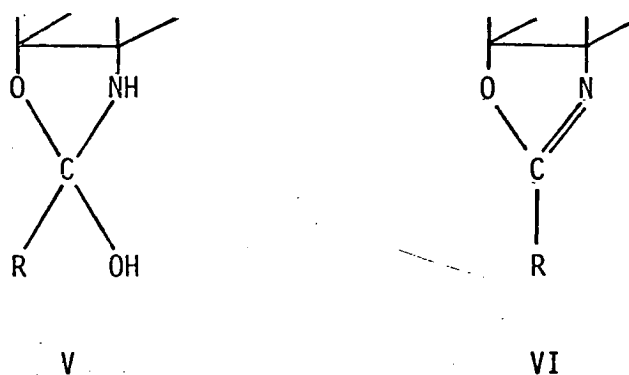
Fig. 8

oxazolines may be formed during migrations.

The migration of the acyl group in ceramides to either the C1-
or the C3-hydroxyl, via oxazolines or oxazolidines, is a possibility
which has not been investigated. Past work has suggested that
during acid hydrolysis of natural sphingolipids, some acyl migration
to the C3-hydroxyl occurs via 2-hydroxy oxazolidine intermediates
(cf. V, Fig. 8)[25,26]. Inversion at C3 during acid hydrolysis is
known to occur since t-sphingoids may be isolated[27,28]. The migration
is thought to proceed through the inversion mechanism (Fig. 7), which
has more recently been verified[18]. In sphingolipids, there are two
hydroxy groups to which the acyl group may migrate; also the C3-
migration may be affected by either the allylic double bond or the
long-chain alkyl group of the sphingoid. As yet, O-acyl sphingoids
have not been prepared. Oxazolines[25,29] and oxazolidines[30], derived
from De-d18:0 or De-18:1, and containing a phenyl group in the 2-
position on the ring, have been successfully prepared.

An acyl migration of a different nature was noticed during the
attempted dephosphorylation of sphingomyelin with acetic acid-acetic
anhydride[31]; similar migrations were observed in glycerophosphatides[32].

This type of migration was thought to be a carbon-to-carbon acetoxyl shift rather than a nitrogen-to-oxygen acyl shift[31].

Anhydrides (e.g. Figs. 4,5) derived from N-acyl dihydroxy sphingoids have not been studied so far as is known. However, in plant materials, tetrahydrofuran derivatives have been produced from phytosphingolipids by dehydration in acid[33,34]; more recently, natural anhydrocerebrins have been found[35].

CHAPTER 1

MATERIALS AND METHODS

1. <u>Materials and equipment</u>

    (a) <u>Lipids</u>

    The main suppliers of materials are abbreviated, KL, APS, SIG, and F for Koch-Light Laboratories, Ltd., Applied Science Laboratories, Inc., Sigma Chemical Co., and Fluka AG, ·Chemische Fabrik, respectively. Others less frequently referred to will be mentioned at the appropriate places.

    Lipids from commercial sources are listed (Table 1).

    Stereoisomers of sphingenine (D<u>e</u>-, DL<u>e</u>, and DL<u>t</u>-isomers) were prepared from brain sphingolipids.

    Pure DL<u>t</u>-sphinganine was prepared from the commercially available compound, which contained some <u>e</u>-sphinganine as an impurity.

    Glucosylceramide and lactosylceramide were obtained from milk sphingolipids.

    Synthetic ceramides were prepared as described by others[36].

    (b) <u>Other chemicals</u>

    Amino acids of greater than 99% purity were obtained from SIG.

    N-ethyl-N'-dimethylaminopropyl carbodiimide was obtained from Serva Biochemicals Inc., 1-fluoro-2,4-dinitrobenzene from KL, and aliphatic nitriles from Aldrich Chemical Co., Inc..

    The reagents for silylation, bis(trimethylsilyl)trifluoro-acetamide, trimethylchlorosilane and SIL-PREP were supplied by APS.

Table 1.   Commercial preparations

1.   Ceramides, from bovine brain cerebrosides (KL)

2.   NFA ceramides, from brain cerebrosides (APS,SIG)

3.   HFA ceramides, from brain cerebrosides (APS,SIG)

4.   Ceramides, from bovine brain sphingomyelin (SIG)

5.   Ceramides, natural mixture (APS)

6.   N-palmitoyl or N-lignoceroyl sphinganine (Miles-Yeda, Ltd.)

7.   Cerebrosides, from bovine brain, highly purified (KL)

8.   Cerasine (N-lignoceroyl-DL-sphingenyl-β-D-galactoside) (SIG)

9.   Sphinganine, rac synthetic (KL)

10. Sphinganine, e or t synthetic (SIG)

11. 4D-hydroxy-De-sphinganine, from yeast (SIG)

12. Sphingenine sulphate, from bovine brain sphingolipids (KL)

13. Sphingomyelin, from bovine brain (KL,SIG)

14. Cephalin, natural (egg yolk) or L-α- and DL-α-synthetic (F)

15. Lecithin, L-α- and DL-α-synthetic (F)

16. Phosphatidyl ethanolamine, synthetic (KL)

17. Phosphatidyl-L-serine, from bovine brain, pure CHR (KL)

18. Lysolecithin (KL)

19. Cholesterol and cholesterol esters (F)

20. Glycerides, synthetic (SIG,APS)

21. Fatty acids and methyl esters, non-hydroxy type (SIG)

22. Methyl esters, branched chain (APS)

23. Methyl esters, 2-hydroxy type (APS)

24. Fatty acids, 2-hydroxy type (F, Serva Biochemicals Inc.).

(c) <u>Chromatographic materials and equipment</u>

Silica gel G (and GF_{254}) and commercially prepared plates were supplied by E. Merck.

Silicic acids for column chromatography were supplied by SIG (SIL-LC type) and Mallinckrodt Chemical Works (100-120 mesh powder).

Gas Chrom Q and liquid phases (GE SE-30 (GC grade), OV-1, OV-7, OV-17, and EGSS-X) were obtained from APS.

TLC tanks were supplied by Camag Inc., and the plate spreader by Shandon Southern.

(d) <u>Instruments</u>

The GLC analysis of methyl esters was carried out on a Phillips PV4000 gas chromatograph, while sphingoids, ceramides, and amino acids were analysed on a Shimadzu GC-4BMPFE instrument; detection in both cases was by means of flame ionisation.

A Hilger-Watts spectrodensitometer was modified by Mr. A. Bottomley, Research Officer, University of Tasmania, for the scanning of TLC plates. Photography was done with a Polaroid overhead camera.

IR, UV, MS, and NMR spectra respectively were recorded on the following instruments:

(i) Beckmann IR-33 spectrophotometer

(ii) Hitachi Perkin-Elmer 124 spectrophotometer

(iii) EAI QUAD-300 mass spectrograph

(iv) JEOL JNM-4H-100 spectrophotometer.

2. Methods of isolation from natural sources

(a) General extraction procedures

For the various tissues and fluids studied, the choice of extraction procedure depended on the weight given to such factors as quantitative recovery of lipids and solvent economy. Small scale operations were carried out by a modification[37] of the Folch procedure[38], while the Bligh and Dyer method[39] was preferred for large scale extractions. In either case, traces of aqueous phase were removed by filtration through silicone-treated (Whatman 1-PS) paper. In general, 95-99% recovery of the lipids was achieved; the losses were due mainly to gangliosides and some polar glycolipids.

(b) Preparation of milk sphingolipids

The extraction of the lipids from whole milk requires large volumes of solvent[39]. An alternative procedure of freeze-drying followed by extraction avoided this problem, and the milk could be stored before extraction.

The sphingolipids are preferably obtained from skim milk[40], although some polar lipids are concentrated in the fat globule membrane[41]. In the following procedure, whole fresh milk from Friesian cows was used.

(i) Extraction of the total lipids

Freeze-dried milk powder (155 g), containing about 35% lipid, was treated with four equal lots (1ℓ) of chloroform-methanol (2:1) and the combined extract partitioned with water (1ℓ)[38]. After standing overnight, the chloroform layer was dried over anhydrous magnesium sulphate, and the solvent removed under reduced pressure at 40°.

(ii) <u>Preparation and fractionation of the polar lipids</u>

The removal of the bulk of the neutral lipids (glycerides, cholesterol esters, and cholesterol) was carried out by liquid-liquid partition[42]. The residual lipids (50 g) were dissolved in the upper phase (120 ml) of a petroleum ether (40-60)-(87%) ethanol equilibrated mixture (50:50). This solution was then extracted twenty times with the lower ethanol phase (50 ml each). Monitoring by TLC showed that the phospho- and glyco-lipids were completely partitioned into the ethanol phase after six extractions. Ceramides, and other lipids of similar polarity required a further fourteen extractions for 95% of their removal. The combined ethanolic extracts were diluted with benzene and concentrated under reduced pressure to yield a lipid fraction containing about 30% phospholipids. The analysis of extracts was carried out by previously tested methods[40].

The separation of the phospho- and glyco-lipids from the neutral lipids and ceramides was carried out by column chromatography. Silicic acid, pretreated by washing out the fines with water[43], was activated at 120°C for 2 hours immediately before use. The powder (100 g), slurried with diethyl ether (240 ml), was used to prepare the column (19 cm x 3½ cm), as recommended for the isolation of lipid classes from 1-2 g of a mixture[44].

The polar lipid fraction (1½ g) was dissolved in a small volume of diethyl ether, and applied to the top of the column. The neutral lipids were eluted (2-3 ml/min) with diethyl ether (400 ml) and discarded. The fraction containing ceramides was eluted with chloroform (150 ml) followed by chloroform-methanol (9:1, 100 ml), and set aside for further analysis (Fraction I). The phospho- and glyco-lipids were eluted with an increasing concentration of methanol in

chloroform (Fraction II), and subjected to mild alkaline hydrolysis to cleave ester bonds[45]. The sphingolipids were then isolated by preparative TLC. Cerebrosides and diglycosylceramides (yields, 12 mg each) gave positive anthrone[46] and negative phosphate[47] tests. Some anthrone-positive material between the sphingomyelin and diglycosylceramide was observed in the position corresponding to sulphatides; on purification by TLC this fraction gave a stronger anthrone test, but there was not enough material for characterization. A third anthrone-positive peak in the elution pattern of buttermilk phospholipids was suggested to be triglycosylceramides[48], by noting some similarities with blood serum glycosphingolipids[49]. The possibility of sulphatides was not mentioned[48], but these compounds could be separated from oligoglycosylceramides if required[50].

(c) Preparation of brain sphingolipids

Brain, second only to spinal cord, is the richest source of natural sphingolipids[51-53], which comprise about 25% of its dry weight. The white matter contains cerebrosides, sphingomyelin, and sulphatides in the ratio 6:2:1, and is an excellent source of d18:1[31,54]. The grey matter contains gangliosides and diglycosylceramides as well, and these contain relatively large quantities of d20:1[31,55].

The dehydration and extraction of much of the cholesterol from a human brain was carried out with acetone essentially as described[51]. Ester-bound glycerophosphatides were hydrolysed with mild alkali[45], and the sphingolipids recrystallised to remove some of the ether-bound glycerophosphatides. The procedures of using ether to remove glycerophosphatides[51], and iodine in potassium iodide to cleave ether linkages[56], were avoided as they lead to losses of sphingolipids.

(i) Dehydration of the brain

Brain tissue (1.5 kg) was homogenised in a Waring blender and stirred overnight with acetone (2 ℓ) at 4°. After centrifuging, the supernatant was poured off, and the solid treated overnight with more acetone (2 ℓ). After decantation of the solvent the brain was dehydrated sufficiently to make filtration possible. The solid was re-extracted four times with acetone (1 ℓ each) to yield a light brown solid (about 270 g). TLC of the acetone extracts revealed the presence of large quantities of cholesterol and other neutral lipids, with small quantities of glycolipids.

(ii) Removal of glycerophosphatides

The dry, solid residue obtained from the acetone dehydration was extracted with 2 lots, 1 ℓ each, of hot chloroform-methanol (2:1), then with the same volume of hot chloroform-methanol (1:2). The combined extracts were taken to dryness under reduced pressure, and the lipid residue (130 g) refluxed for 10 minutes in dry methanol (1 ℓ) containing fresh sodium (5 g). The solution was neutralised to phenolphthalein with anhydrous methanolic hydrogen chloride (1 N, about 220 ml), chloroform (2.5 ℓ) added and partitioned with water (1 ℓ). Salts and glyceride degradation products in the upper phase were removed by aspiration, and the lower phase evaporated to dryness (yield, 108 g). Recrystallisation from ethanol gave an off-white powder (70 g), consisting essentially of sphingolipids. The removal of neutral lipid contaminants with non-polar solvents such as hexane or carbon tetrachloride resulted in a colloidal sphingolipid suspension which could not be centrifuged successfully. The preparation still contained small amounts of methyl esters, and degradation products of the plasmalogens and lyso-ethers. These could be removed, and the separation of the sphingolipids effected

by column chromatography[56].

(iii) <u>Ceramides from brain sphingolipids</u>

With the exception of ceramides, sphingolipids are only partially hydrolysed with alkali, and undergo acid hydrolysis with difficulty resulting in relatively low yields of sphingoids and undesirable byproducts. Thus, a method for the conversion of total sphingolipids to ceramides would be advantageous. Sphingomyelins may be converted to ceramides by treatment with acetic acid-acetic anhydride[57] or phospholipase C[58]. Sulphatides may be converted to cerebrosides by treatment with acetic acid[59] or anhydrous methanolic hydrogen chloride[60]. In the author's experience the acetic acid-acetic anhydride treatment[57] was preferred owing to the simultaneous removal of the phosphorylcholine moiety from sphingomyelins and the sulphate group from sulphatides. A disadvantage of this procedure was that some hydrolysis of the amide bond occurred, producing diastereoisomeric N-acetyl sphingoids.

Total brain sphingolipids (10 g) were heated with acetic acid-acetic anhydride (4:1, v/v; 100 ml) under reflux for one hour. The solvent was removed under reduced pressure, and the residue treated with chloroform-0.6 N methanolic sodium hydroxide (1:1, v/v; 1ℓ) for one hour at room temperature[45]. Hydrochloric acid (0.6 N, 500 ml) was then added, the mixture shaken vigorously and the chloroform phase removed. This solution contained chiefly cerebrosides with smaller amounts of ceramides and N-acetyl sphingoids. The solvent was removed under reduced pressure (8.6 g of lipid recovered), and the cerebrosides were degraded to ceramides by the procedure of Carter, Rothfus and Gigg (1962)[61].

(iv) Preparation of sphingoids

The stereoisomers of sphingenine and sphinganine have been chemically synthesised[62-64]. However, the configuration of the sphingoids isolated from natural products can be determined by the hydrolytic procedure used. Acid or alkaline hydrolysis of t-ceramides produces t-sphingoids only (see Ch. 3, p. 90). Alkaline hydrolysis of e-ceramides produces e-sphingoids only[65], but acid hydrolysis produces a diastereoisomeric mixture, with the e-isomers predominating[27,57]. To obtain the best yield possible of t-sphingoids by existing hydrolytic procedures, with a minimum of by-products , aqueous-methanolic hydrochloric acid (1 N) could be used[66].

Alkaline hydrolysis[61] of the ceramides from (iii) yielded essentially De-sphingoids (2.3 g, from 10 g of sphingolipids); small amounts of t-sphingoids, detected as DNP derivatives[31], were found as a result of the dephosphorylation treatment. Recrystallization from ethyl acetate gave a light yellow wax, m.p. 60-70° (1.8 g). Its composition, determined by GLC (see p. 36, this chapter), was e-d18:1 (86%), t-d18:1 (8%), e-d18:0 (3%), and other sphingoids (3%).

Acid hydrolysis of the ceramides[66], followed by N-acetylation of the sphingoids[67], produced a mixture of mainly three derivatives which were separated by TLC on borate-impregnated TLC plates (solvent, chloroform-methanol (8:1)). The derivatives were characterised by IR spectroscopy, mass spectroscopy, and GLC; the sphingoid composition was thus determined as e-d18:1 (40%), t-d18:1 (33%), e-d18:0 (2%), and 3-O-Me-d18:1 (25%). The pure, isomeric sphingoids were obtained after alkaline hydrolysis[65]. A comparison of the sphingoid composition obtained after the above treatment, with that obtained after acid hydrolysis[66] of pure cerebrosides, was made. The sphingoid composition was, in the latter case, e-d18:1 (58%), t-d18:1 (20%),

<u>e</u>-d18:0 (2%), and 3-O-Me-d18:1 (20%).

3. <u>TLC Methods</u>

The versatile technique of TLC was used for the fractionation of natural lipid mixtures, also for the assessment of the purity of lipid preparations and the identification of individual constituents. It was also used for the monitoring of extractions and separations of columns.

For the purposes of this work, the chromatographic adsorbent, silica gel G, was standardised. This is a white powder consisting of silicic acid ($SiO_2.xH_2O$) and contains anhydrous calcium sulphate as a binder. The structure of the former is regarded to consist of tetrahedral siloxane (Si.O.Si) units, with silanol (Si.OH) groups present at the surface[68]. A mono- or multi-molecular layer of adsorbed water covers the surface, but this "free" water can be removed reversibly by heating at 100 to 120°. The amount of free water present in the silica gel was found to influence the chromatographic mobility of compounds such as free ceramides[40].

(a) <u>Preparation of plates</u>

A slurry of silica gel G in distilled water was applied to a metre-long rack of glass plates (20 cm x 5 to 100 cm) by means of a commercial applicator. Analytical plates (250 μ thick) were prepared using a slurry of 33 g of silica gel in 60 ml of water. Preparative plates (1 mm thick) were prepared using a slurry of 100 g of silica gel in 175 ml of water. The plates were air-dried for 30 minutes, followed by activation for 1 hour at 110°.

Chemicals were incorporated by using solutions in the place of

water. Sodium tetraborate (or simply "borate") impregnated plates were often used, and a 10% $^{W}/w$ loading of the chemical on the dry silica gel gave effective separations. These plates were stable indefinitely under normal laboratory conditions. Silver nitrate impregnated plates darkened quickly, and were best used immediately after activation; their preparation required a plastic spreader rather than the conventional brass spreader as the silver ion could displace the copper onto the plate.

(b) Development

Chromatograms were developed in rectangular glass tanks lined with solvent-saturated filter paper[69].

The solvents used for TLC were of analytical reagent grade; acetone and diethyl ether were redistilled. Two mixtures commonly used were

(i) chloroform-carbon tetrachloride-methanol-water (50:50:12:0.5).

(ii) chloroform-methanol-water (65:25:4)[70].

Many of the lipid materials used for TLC standards have been developed in either of these mixtures, and corresponding R_f values tabulated[40].

Solvent (i), useful for the analysis of ceramides and lipids of comparable polarity, gave more reproducible results than the chloroform-methanol mixtures usually used for the TLC analysis of ceramides[71], also the presence of water increased the resolution of NFA and HFA ceramides. Solvent (ii) was used for the analysis of phospho- and glycolipids.

Fresh solvent was necessary after the development of five to six chromatograms, as the more volatile components were lost; also, impurities were transferred from the adsorbent.

(c) <u>Visualisation</u>

The general reagent used for the detection of lipids was a solution of sulphuric acid (50%) containing potassium dichromate (0.5%)[72]. The solution was sprayed as a fine mist onto the plates, and the lipids charred by heating for 30 minutes at 250°.

Lipids containing known functional groups were distinguished with the aid of specific reagents. Sensitive spray reagents are available for the detection of phosphorus[47], sugar[46], nitrogen[73], ester[74], free amino[75], and vicinal diol[76].

The plates were photographed for permanent record.

(d) <u>Recovery of lipids from plates</u>

Non-destructive reagents such as iodine, water, or fluorescent indicators (e.g. dichlorofluoroscein) have been used for detecting lipids on TLC plates prior to their recovery. In this laboratory, eosin was found to be very sensitive to lipids at 254 nm, much more so than dichlorofluorescein. Its limit of detection was 0.5 µg of lipid (as pure ceramide or cholesterol) over 1 cm on an analytical plate. An aqueous solution (0.5%), sprayed as a fine mist onto the chromatogram, was adequate for the purpose.

The portions of silica gel containing the desired compounds were marked under the UV light, scraped off the plate, and treated with chloroform-methanol (2:1). Silica gel and indicator were removed by washing the extract with one-third the volume of a dilute borate solution. The extract was filtered, and the solvent removed under reduced pressure.

Preparative separations of compounds of comparable chromatographic mobility were carried out on layers 250 µ thick, rather than the 1 mm layers conventionally used. The operation was time consuming, but

better separations were obtained (a possible reason for this is the difference in movement of a particular compound either near the surface of the adsorbent or near the glass plate).

(e) Purification of natural ceramides

The TLC analysis of natural ceramides in solvent (i) is rendered difficult by the presence of other lipids of comparable mobility, namely fatty acids and monoglycerides. As demonstrated (Fig. 1), fatty acids move closer to the NFA ceramides, while monoglycerides move with the HFA ceramides. Attempts to wash out the fatty acids from a chloroform-methanol (2:1) solution of the lipids with alkalis such as ammonia, sodium carbonate and sodium hydroxide solutions were ineffective. Neither could the monoglycerides be removed from total lipids by mild hydrolysis[45].

Silica gel plates impregnated with borate or arsenite ions were tested for their effect on the relative mobilities of the lipids, but resolution of major classes was not satisfactory by such methods. Borate retarded slightly the monoglycerides and the fatty acids moved with the NFA ceramides. Arsenite stopped the fatty acids from moving, but the monoglycerides moved with the NFA ceramides.

The problem was solved by 2-dimensional TLC (Fig. 3, p.28).

(i) Removal of the fatty acids

The mobility of fatty acids relative to ceramides was influenced by the presence of alkalis or acids in the developing solvent. The solvent, chloroform-carbon tetrachloride-methanol-15N ammonium hydroxide (50:50:12:1) caused the fatty acids to stay near the origin, while the presence of acetic acid (1-2%) caused them to move close to the solvent front (Fig. 2).

Weak alkali such as sodium carbonate, added to the adsorbent, caused acidic components to remain at the origin. A convenient use of this method was that of applying a narrow band (0.5 cm wide) of the alkali immediately above the origin on a plate. Other lipids were not affected by the barrier, migrating quantitatively to their normal R_f values.

A less convenient method to remove the fatty acids was by treatment with diazomethane. Methyl esters were produced[77], which migrated to the solvent front on the TLC plates. This method was avoided, due to the toxicity of the reagent and its harmful effects on the basic phosphatides.

(ii) Separation of the monoglycerides

In general, chlorinated solvent-alcohol-water mixtures caused monoglycerides and HFA ceramides to move together on the plate (cf. Figs. 1,2). Substituting the chloroform for ether or the alcohol for acetone (or dioxan), influenced the relative mobilities of the ceramides and monoglycerides; the latter now moved with the NFA ceramides. Dry ether-acetone mixtures completely separated the lipids, but ceramide bands appeared diffuse and not as well resolved as they could have been had water been present in the solvent.

The most effective solvent mixture was ether-acetone-15N ammonium hydroxide (30:20:2), which caused the monoglycerides to move near the NFA ceramides. The presence of the aqueous alkali not only removed fatty acids from the TLC system, but increased the resolution of the two ceramide bands. As the second solvent of a 2-D chromatographic system, with chloroform-carbon tetrachloride-methanol-15N ammonium hydroxide (50:50:12:1) as the first solvent, monoglycerides were completely separated from NFA and HFA ceramides (Fig. 3). The
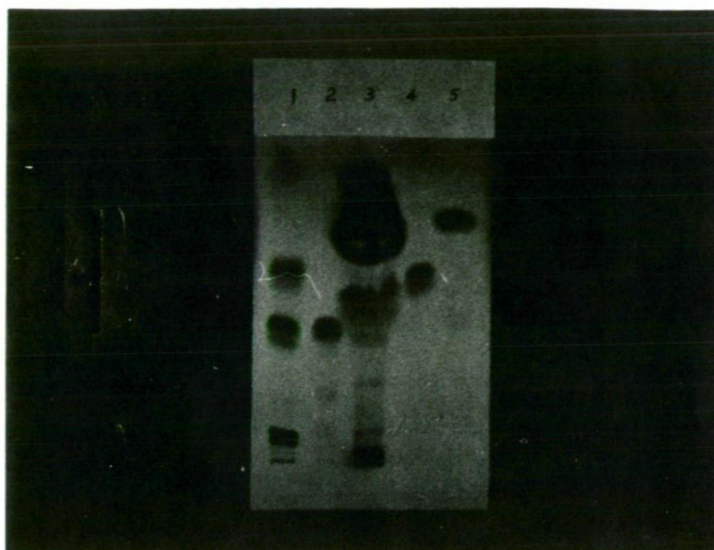
Fig. 1.   TLC plate illustrating the relative mobilities of ceramides and some neutral lipids.   Developing solvent, chloroform-carbon tetrachloride-methanol-water (50:50:12:0.5).   1, NFA ceramides (upper spot), HFA ceramides (middle spot), and cerebrosides (lower spots); 2, α-monoolein; 3, serum lipids; 4, oleic acid; 5,cholesterol.

Fig. 2.   TLC plates illustrating the removal of fatty acids from ceramides and monoglycerides.   Developing solvents for plates A,B, and C respectively were chloroform-carbon tetrachloride-methanol-15 N ammonium hydroxide (50:50:12:1), chloroform-methanol-acetic acid (95:5:2), and chloroform-carbon tetrachloride-methanol-water (50:50:12:0.5).   A sodium carbonate band is present above the origin on plate C.   1, NFA ceramides (upper spot) and HFA ceramides (lower spot); 2, serum lipids; 3, oleic acid.
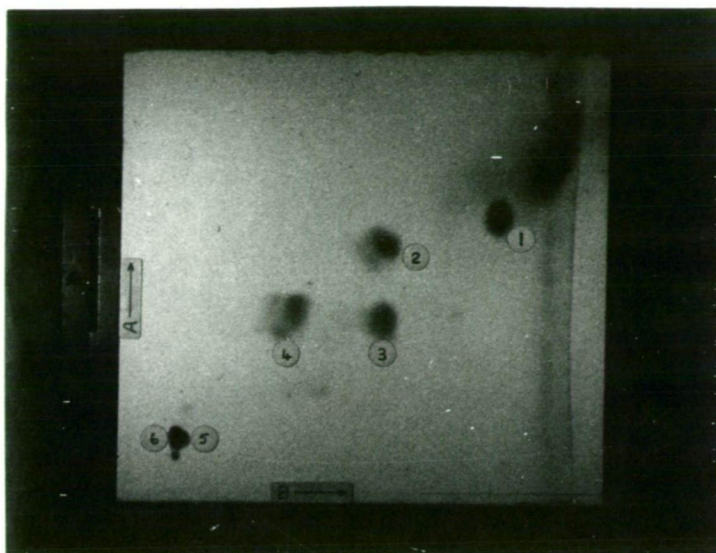
Fig. 3.   2-dimensional TLC plate illustrating the separation of ceramides from monoglycerides.   Developing solvents for directions A and B respectively, chloroform-carbon tetrachloride-methanol-15 N ammonium hydroxide (50:50:12:1) and ether-acetone-15 N ammonium hydroxide (80:20:2).   1, cholesterol; 2, NFA ceramides; 3, α-monoolein; 4, HFA ceramides; 5, oleic acid; 6, cerebrosides.
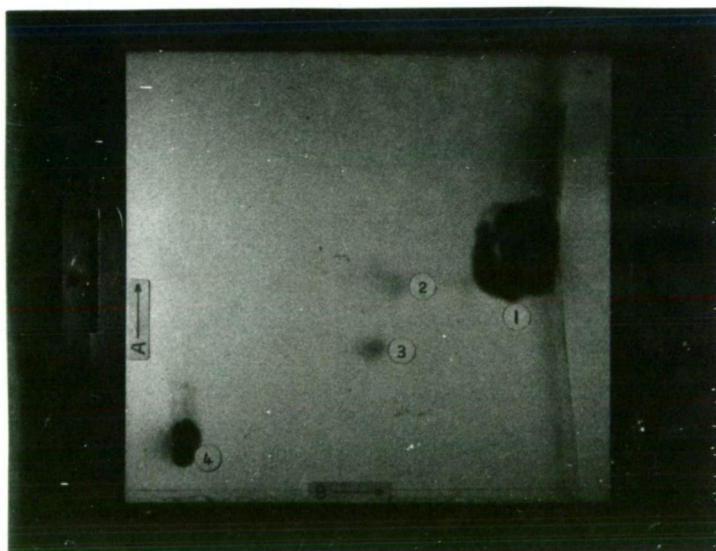
Fig. 4.   2-dimensional TLC plate illustrating the separation of serum lipids.   Developing solvents, as for Fig. 3.   1, cholesterol plus other neutral lipids; 2, NFA ceramides; 3, monoglycerides; 4, fatty acids plus polar lipids.

method was used for the identification of these lipids in blood serum; NFA ceramides and monoglycerides were present, and HFA ceramides were absent (Fig. 4).   Recovery of the fatty acids free from other lipids, could be achieved if desired by replacing ammonium hydroxide with water in the first solvent.

(f) Spectrodensitometry of lipids

The quantitative analysis of lipids may be carried out by spectrodensitometry.   The accuracy is dependent on such factors as variation in thickness and degree of moisture of the adsorbent, the process used to apply the locating reagent, the shape and demarcation of the spots, and the distribution of components[78].

The variations in thickness became insignificant since commercially prepared plates were used.   The moisture level was controlled using desiccated cabinets.

The process used to apply the locating reagent was immersion after TLC[79].   This was found more practical than either spraying after TLC, or incorporation in the adsorbent before TLC[80-82].

The shape and demarcation of the spots were largely controlled by lining the TLC tanks with filter paper.

(i) Analysis of serum lipids using phosphomolybdic acid

Commercially prepared silica gel G plates were prewashed with chloroform-methanol (2:1) in a TLC chamber.   Sodium carbonate barriers were applied just above the origin and the plates dried at 90° for 30 minutes.   Lipids in chloroform were applied evenly over 1.0 cm widths and 1.5 cm apart by means of a microsyringe.   The plates were then developed in a suitable solvent system.   After drying, they were immersed evenly and slowly into a solution consisting

of 5 g of phosphomolybdic acid in 70 ml of water, 25 ml of ethanol, and 5 ml of 70% perchloric acid[79]. The excess reagent was quickly removed with blotting paper, and the plates heated for exactly 20 minutes at 85°. After cooling, the blue phosphomolybdate spots were scanned.

Results obtained by this method were reproducible. Neither the reagent on the plates nor the colour of the spots deteriorated after 4-5 days provided the plates were overlaid with a clean glass plate and stored in the dark. After several weeks, the plates protected with glass gradually assumed a blue colour, while plates exposed to the atmosphere returned to the original yellow colour within days.

Standard calibration curves for cholesterol, ceramides, and monoglycerides are illustrated (Fig. 5). The method was sensitive, with lower limits of 0.05 µg for cholesterol, 0.1 µg for ceramide, and 0.5 µg for monoglyceride. The relationship, area = kCB, where C is the concentration[83], did not fit the durves for cholesterol or ceramides. It will be seen that the densities of the spots vary significantly for the same concentration of the three lipids. This indicates other factors are involved in the reduction of the phosphomolybdic acid besides double bonds. It was shown that a saturated monoglyceride (α-monopalmitin) did not reduce the phosphomolybdic acid, either alone or when in the presence of natural monoglycerides or HFA ceramides. Thus hydroxyl, ester, and amide groups have no effect on the reagent under the conditions described. Tripalmitin and stearic acid showed no reaction either[79].

In practice, cholesterol esters, triglycerides, diglycerides and cholesterol, are simply separated by TLC, and assayed by spectro-densitometry. The analysis of ceramides or monoglycerides is more difficult, since spectrodensitometry measures the total concentration
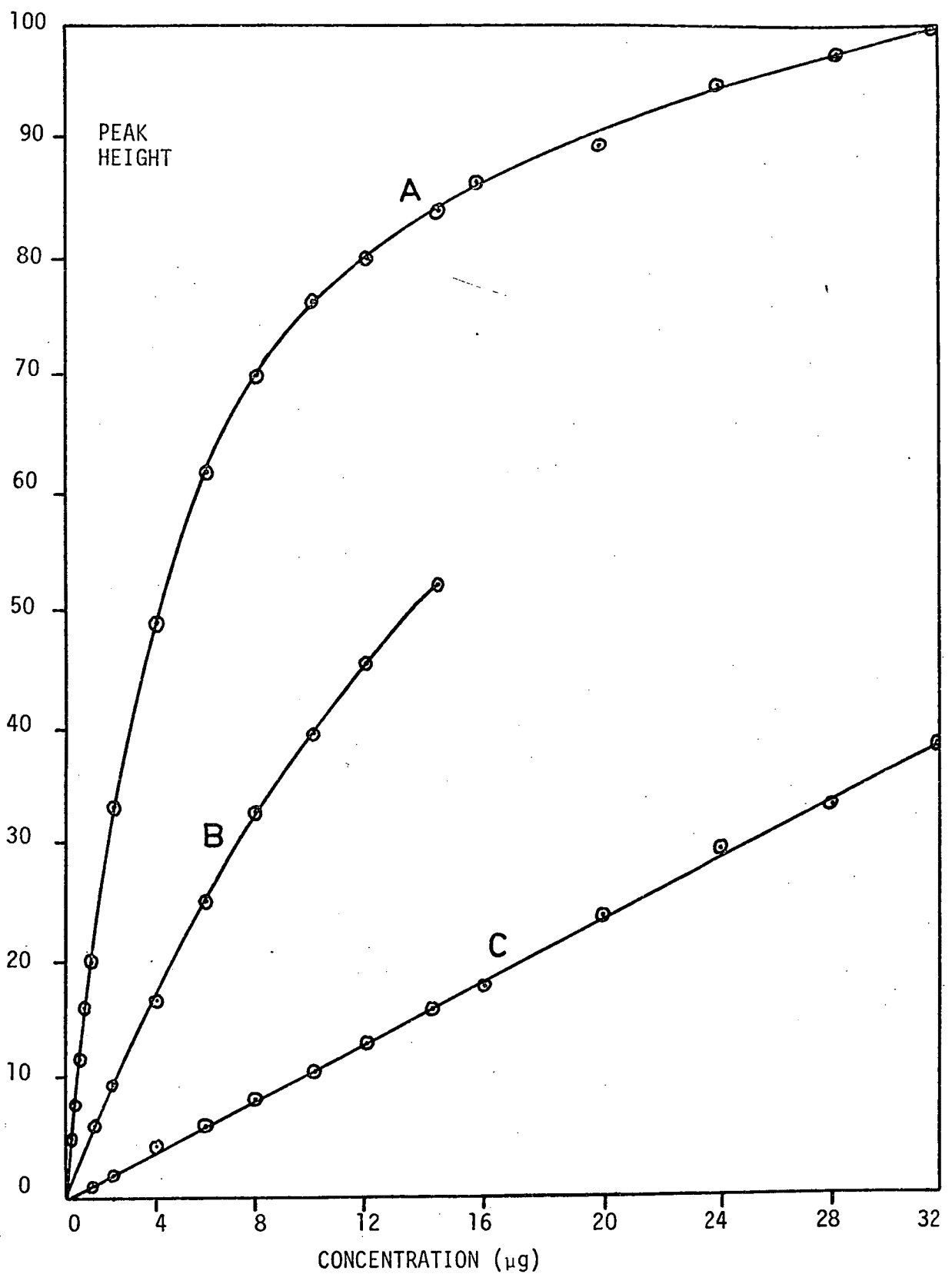
Fig. 5. Spectrodensitometric analysis of cholesterol (curve A), ceramides (curve B), and monoglycerides (curve C) with phosphomolybdic acid.

of these lipids which have similar chromatographic mobilities. To overcome this difficulty, one must look either to specific reagents or solvents that allow sufficient chromatographic separation.

(ii) Analysis of serum monoglycerides

Serum monoglycerides were analysed by spectrodensitometry of the coloured spots produced by their reaction with sodium metaperiodate and Schiff's reagents[76]. The colours did not deteriorate on standing for some time, and since the time for scanning was less than 1 minute, the results were reliable.

A known quantity of normal serum lipids were applied to a commercially prepared plate. Milk monoglycerides, consisting mainly of monoolein, were used as standards. The plate was developed in chloroform-methanol (93:7), dried, and immersed slowly and evenly into a solution of sodium metaperiodate (1%). After 30 seconds, excess reagent was quickly removed with blotting paper, and the plate placed in a tank saturated with dry sulphur dioxide for 15 minutes. It was then sprayed lightly with Schiff's reagent and placed back into the tank for 1 hour. The coloured spots due to p-rosaniline were scanned immediately after removal from the sulphur dioxide atmosphere. The value, 5 mg %, was a typical value for normal serum monoglycerides[79].

(iii) Analysis of serum ceramides

Serum ceramides were analysed by spectrodensitometry of the coloured spots produced by their reaction with t-butylhypochlorite and potassium iodide-starch reagents[73]. The plates darkened within minutes on exposure to the atmosphere, and iodine was lost from the spots. However, consistent results were obtained with standards subjected to the same conditions provided that analysis was carried

out without delay.

A known quantity of the serum lipid was applied to a silica gel plate, prepared as described previously; commercially prepared plates were found unsuitable for this method. NFA ceramides were used as standards. The plate was developed in chloroform-methanol (93:7), dried, and sprayed evenly with a solution of t-butylhypochlorite in cyclohexane (2%). After drying under a cold air blast for 1 hour, the plate was sprayed lightly with a solution of KI-starch (1%) and the coloured spots scanned immediately. Values of 1 mg% for NFA ceramides and 0.04 mg% for HFA ceramides were obtained.

4. GLC Methods

GLC was used for three purposes, for long-chain methyl ester, N-acyl sphingoid, and amino acid analyses.

(a) Analysis of methyl esters

Methyl esters, both saturated and monounsaturated, were analysed by GLC on 3% GE SE-30 (GC grade) on Gas Chrom Q contained in silanized glass columns (3 mm ID by 2 m). Polyunsaturated methyl esters were distinguished on a column of 3% EGSS-X on Gas Chrom Q. Columns were conditioned for 36 hours at temperatures near the upper limits for the liquid phases.

GLC operating conditions

Initial temperature, 160°

Final temperature, 310°

Programming rate, 5°/minute

N_2 and H_2 flow rates, 20 ml/minute

Air flow rate, 250 ml/minute

Detector temperature, 310°

Injector temperature, 310°

Using the above operating conditions, the relative retention times of some unsubstituted methyl esters (A), 2-trimethylsiloxy methyl esters (B), and 2-acetoxy methyl esters (C) have been recorded (Table 2). The direct relation observed between the carbon number and relative retention time (Fig. 6) permits extrapolation or interpolation so as to determine the retention times of other methyl esters.

Table 2

Retention times of methyl esters, relative to methyl stearate (18:0)

Carbon no.	A	B	C
12:0	0.27	-	-
14:0	0.47	0.77	-
16:0	0.73	1.02	0.19
18:0	1.00	1.29	0.38
20:0	1.27	1.55	0.59
22:0	1.53	1.78	0.86
24:0	1.78	2.01	1.13
26:0	2.07	2.23	1.34
18:1	1.05	-	-
18:2	0.96	-	-
18:3	0.96	-	-
20:1	1.23	-	-
22:1	1.50	-	-
24:1	1.75	2.20	-
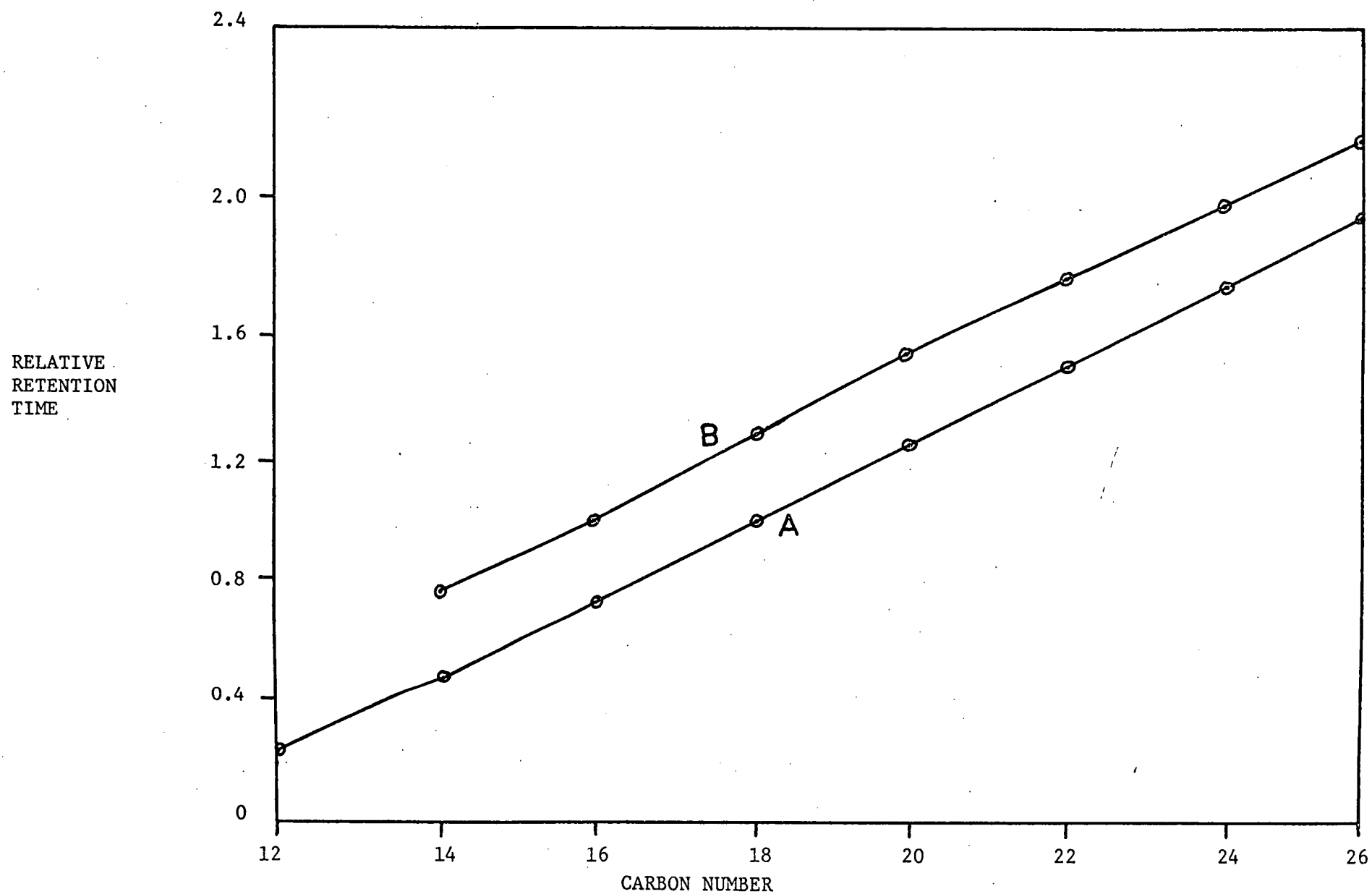
Fig. 6.   Retention times of saturated normal methyl esters (curve A) and 2-trimethylsiloxy methyl esters (curve B), relative to methyl stearate.

(b) <u>Analysis of N-acyl sphingoids</u>

N-acyl sphingoids (ceramides), including N-acetyl sphingoids,
were analysed as their TMSi ethers by GLC on a column (3.3 mm by 3 m,
glass) containing 1% GE SE-30 (GC grade) plus 1% OV-17 on Gas Chrom Q.
A column of 1% OV-1 on Gas Chrom Q, which has been favoured for
separating homologues of <u>e</u>-ceramides[84], gave equally effective
separations.   The relative retention times of diastereoisomeric
ceramides were similar to those observed for the N-acetyl analogues,
separated on the above columns, or on 3.8% SE-30 on Diatoport S[85]
(a study of stereoisomers is given in Ch. 3).

Derivatisation of the N-acyl sphingoids prior to GLC was
accomplished by treatment for one hour with SIL-PREP (200 μg of lipid
was treated with 100 μℓ of reagent).   N-acetyl derivatives were
analysed in the reagent, while ceramide derivatives were analysed in
carbon disulfide after removing the reagent under reduced pressure
with an oil pump [86].

The carrier gas used was helium rather than nitrogen, since this
has been found to increase the resolution of ceramide derivatives,
and give a better relative detector response[84].

<u>GLC operating conditions</u>

Column temperatures were 220° or 300°, for N-acetyl sphingoid or
ceramide derivatives, respectively (the latter could be separated on
1% OV-1 on Gas Chrom Q at 280° with similar retention times).

Detector temperature, 280°
He flow rate, 50 ml/min.
H$_2$ flow rate, 40 ml/min.
Air flow rate, 300 ml/min.

Table 3

Retention times of TMSi derivatives of diastereoisomeric N-acyl

sphingoids, relative to the isomer derived from e-d18:0

Sphingoid moiety in derivative	N-acetyl derivative	N-stearoyl derivative
t-d18:1	0.84	0.85
t-d18:0	0.87	0.89
e-d18:1	0.93	0.93
e-d18:0*	1.00	1.00
3-0-Me-d18:1	1.10	
2D,3D,4D-t18:0	1.20	

*The actual retention time for the derivatives containing e-d18:0 was

about 16.5 minutes.

(c) Analysis of amino acids

Amino acids were converted to their TMSi derivatives, and analysed

by GLC essentially as described[87]. The column consisted of 10% OV-7

on Gas Chrom Q contained in glass columns (3.3 mm ID by 3 m), and gave

separations similar to those obtained on a column containing less

liquid phase.

GLC operating conditions

Initial temperature, 75°.
Final temperature, 290°.
Programming rate, 4°/minute.
N_2 flow rate, 60 ml/minute.
H_2 flow rate, 40 ml/minute.
Air flow rate, 300 ml/minute.
Detector temperature, 280°.

In the accompanying table (Table 4), retention times are given relative to phenanthrene. The structures of amino acid derivatives have been recorded[87]. Amino acids that can contain 2,3 or 4 TMSi groups are given numbered subscripts.

Table 4

Retention times of TMSi derivatives of amino acids, relative to phenanthrene

Amino acid derivative	Relative retention time
Alanine	0.36
Glycine$_2$	0.39
Valine	0.47
Leucine	0.52
Isoleucine	0.55
Glycine$_3$	0.56
Proline	0.57
Serine	0.61
Threonine	0.63
Hydroxyproline	0.74
Aspartic acid	0.75
Methionine	0.76
Cysteine	0.78
Arginine$_3$	0.78
Glutamic acid$_2$	0.78
Glutamic acid$_3$	0.82
Phenylalanine	0.84
Lysine$_3$	0.92
Arginine$_4$	0.95
Lysine$_4$	0.98
Histidine$_2$	1.01
Tyrosine	1.03
Histidine$_3$	1.05
Tryptophan$_3$	1.21
Cystine	1.25
Tryptophan$_2$	1.25

CHAPTER 2

LIPIDS ASSOCIATED WITH OR DERIVED FROM CERAMIDES

1. Polyol artefacts in KL ceramides

Ceramides, prepared from mono-, di-, or trihydroxy e-sphingoids, and normal or racemic 2-hydroxy fatty acids, have been analysed by TLC[71,88]. Natural ceramides, differing in the number, position, and stereochemistry of hydroxy groups[89,90] and the fatty acid chain length[91], have also been studied. Silver ions added to the adsorbent causes the separation of ceramides differing in the degree of unsaturation[45,92]; for distinguishing between derivatives containing either e-d18:1 or e-d18:0, borate has been used[93]. Observations of chromatographic spots that could not be interpreted on findings listed above gave rise to investigations reported in this section.

The low R_f values suggested polar groups in excess of those normally found in ceramides. Of particular interest were two compounds with R_f values of 0.25 and 0.20 (Fig. 1, lane 2) observed in some KL ceramide preparations . . The polyols described by Hammarström[92] have similar R_f values, but other possibilities cannot be excluded. Serum lipids in sufferers from heart disease have constituents of the same chromatographic mobility; these were considered to belong to a class of ceramides with additional hydroxy groups on either the fatty acid or sphingoid moeities[2]. Ceramides derived from t18:0 belong to this class (lanes 3 and 4), and these have been found in small concentration in animals[90].

A sample of the KL ceramide was separated by preparative TLC into the four lipid classes tentatively regarded as "ceramides 1,2,3 and 4" in order of decreasing R_f values[94]; the solvent for TLC was chloroform-
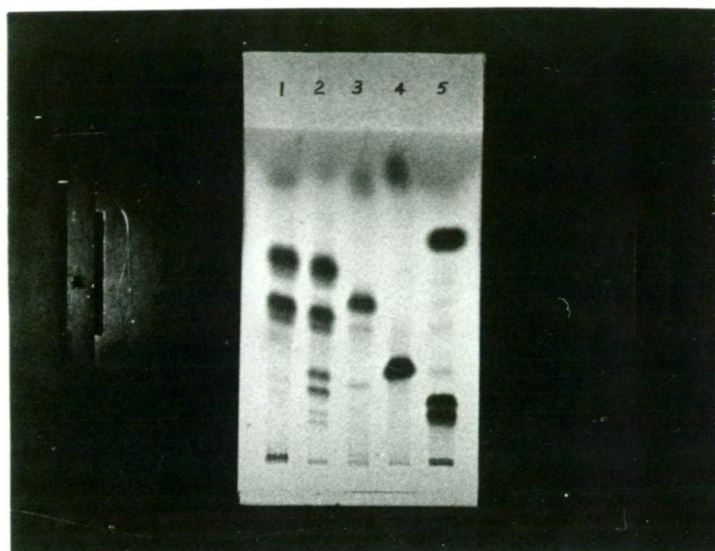
Fig. 1.   TLC plate illustrating the relative mobilities of
ceramides and cerebrosides.   Developing solvent, chloroform-carbon
tetrachloride-methanol-water (50:50:12:0.5).   1, KL ceramides, pure;
2, KL ceramides (batch No. 43209), with the show-moving compounds;
3, 24:0-t18:0; 4, D-h16:0-t18:0; 5, cholesterol (upper spot) and
cerebrosides (lower spots).

methanol (92:8). The following methods (a) to (e) proved that "ceramides 1 and 2" were respectively NFA and HFA derivatives of d18:1, and that "ceramides 3 and 4" were respectively NFA and HFA derivatives of the sphingoid

$$CH_3(CH_2)_{12}CH{=}CH{-}\underset{\underset{OH}{|}}{CH}{-}\underset{\underset{NH_2}{|}}{CH}{-}CH_2{-}O{-}CH{-}O{-}\underset{\underset{CH_2OH}{|}}{CH}{-}CH_2OH$$

with CH$_2$OH and CH$_2$OH substituents
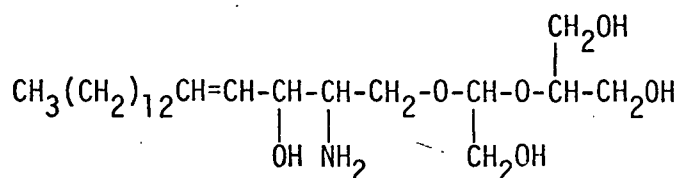
(a) <u>IR spectroscopy</u>

The IR spectra of the four ceramide classes were alike; "ceramides 3 and 4" showed a stronger ν C-O(1050-1040 cm^{-1}) which indicated a higher content of primary hydroxyl groups. The C-H out-of-plane deformation (960 cm^{-1}) for the <u>trans</u> double bond indicated the presence of d18:1.

(b) <u>Conversion of cerebrosides to polyols</u>

Degradation of KL cerebrosides[61], followed by TLC[92], afforded polyols which had the same chromatographic mobilities as "ceramides 3 and 4". Mild acid hydrolysis[61] of these compounds produced "ceramides 1 and 2" respectively.

(c) <u>Analysis of the sphingoids by mass spectroscopy</u>

Strong alkaline hydrolysis of the ceramides[93] yielded sphingoids, which were converted to TMSi-N-acetyl sphingoids[67]. The mass spectra for the derivatives obtained from "ceramide 1" (Fig. 2), "ceramide 2" (not shown), and KL sphingenine (Fig. 3) were essentially identical. With the exception of minor peaks due to 1,3-di-O-TMSi-N-acetyl d18:0 (compared with a pure sample in Fig. 4), the spectra were similar to the published one for 1,3-di-O-TMSi-N-acetyl d18:1[67]. The absence of

peaks due to 1,3,4-tri-0-TMSi-N-acetyl t18:0 was verified by comparison of the spectra with a published one for this derivative[95]. The mass spectral fragmentation modes for the above derivatives follow those proposed for other TMSi-N-acyl sphingoids[84,96], and will not be discussed here.

The spectra for the derivatives obtained from "ceramide 3" (Fig. 5) and "ceramide 4" (not shown) have a prominent peak at m/e 311 which also occurs for the derivative obtained from d18:1 (Fig. 3). Some other fragmentation modes are represented in Fig. 5, together with the structure, for these derivatives. Two mechanisms which account for some of the observed ions have been ascribed to the effects of the radical and positive ion sites, respectively[97]. If A represents the sphingenine part of the molecule, and B the polyol part, then the two mechanisms may be represented as follows:

$$A-CH_2-\overset{+}{O}\cdot-B \longrightarrow A\cdot + CH_2=\overset{+}{O}-B \quad \ldots\ldots (i)$$

$$A-CH_2-\overset{+}{O}\cdot-B \longrightarrow A-CH_2-O\cdot + B^+ \quad \ldots\ldots (ii)$$
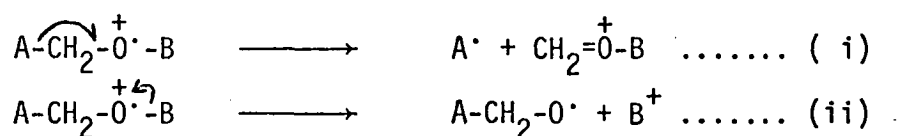
Ions at m/e 396 and m/e 398 for the sphingenine and sphinganine derivatives respectively may thus be proposed.

$$\text{R-CH-CH-CH}_2\text{-O-CH}< \longrightarrow \text{R-CH-CH-}\overset{\cdot}{C}H_2 + \overset{\cdot}{O}\text{-CH}<$$

with RTMSiO and NH substituents (NH bearing COCH$_3$) on both sides.
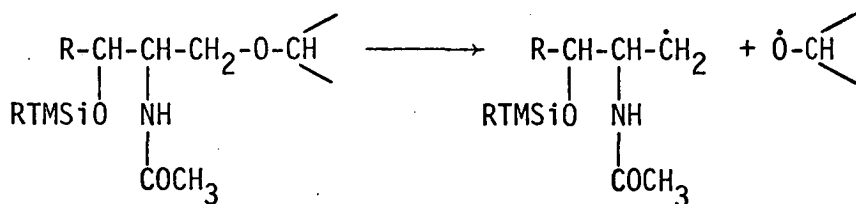
Other ions due to mechanism (ii) occur at m/e 351, m/e 319, m/e 396 or 398 and m/e 262 (the latter due to loss of trimethylsilanol from the ion at m/e 351). An ion expected at m/e 452 for cleavage between C_2 and C_3 of the sphingenine moeity, with charge retention on the fragment containing the acyl moeity, does not appear. However the ions at m/e 306 and m/e 216 may result from secondary rearrangements,
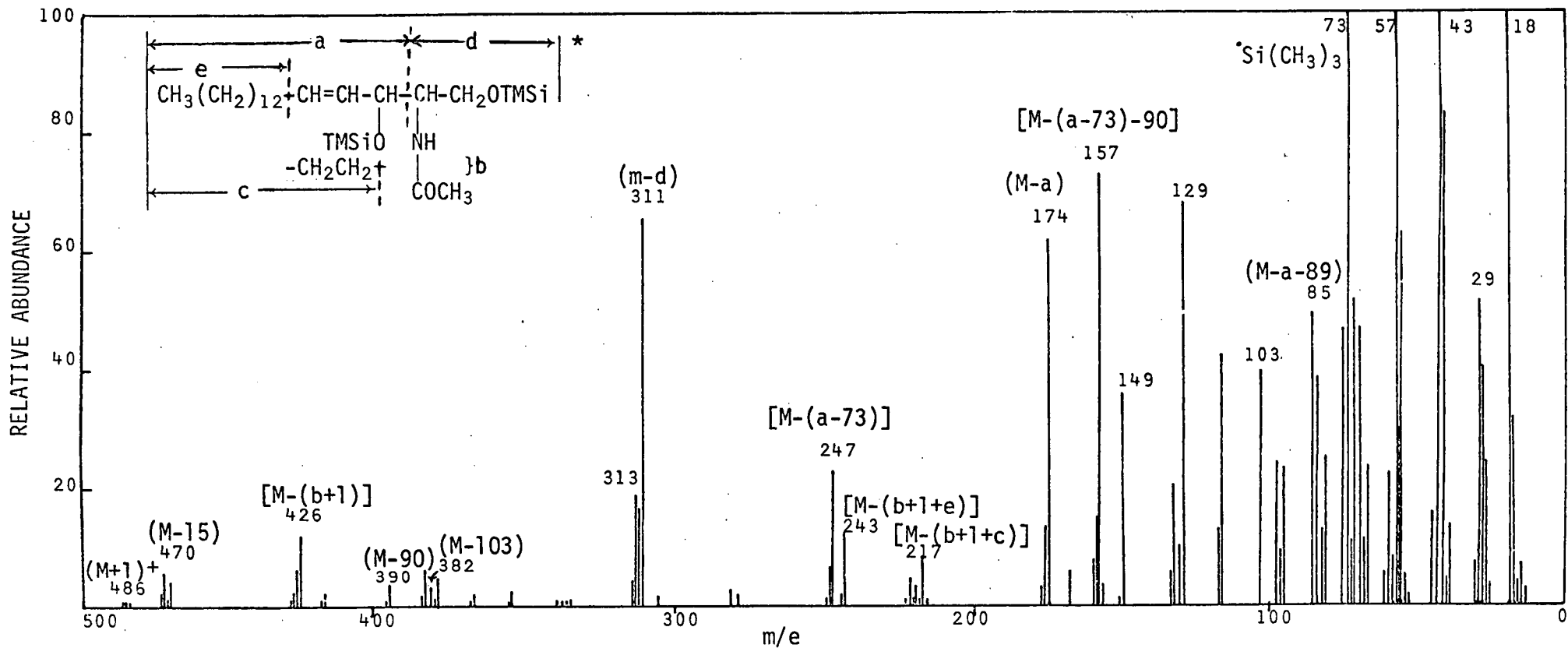
Fig. 2.   Mass spectrum of the di-O-TMSi-N-acetyl sphingoids derived from KL "ceramide 1".

*The symbols used to indicate fragments are the same as those used by Samuelsson and Samuelsson[84].

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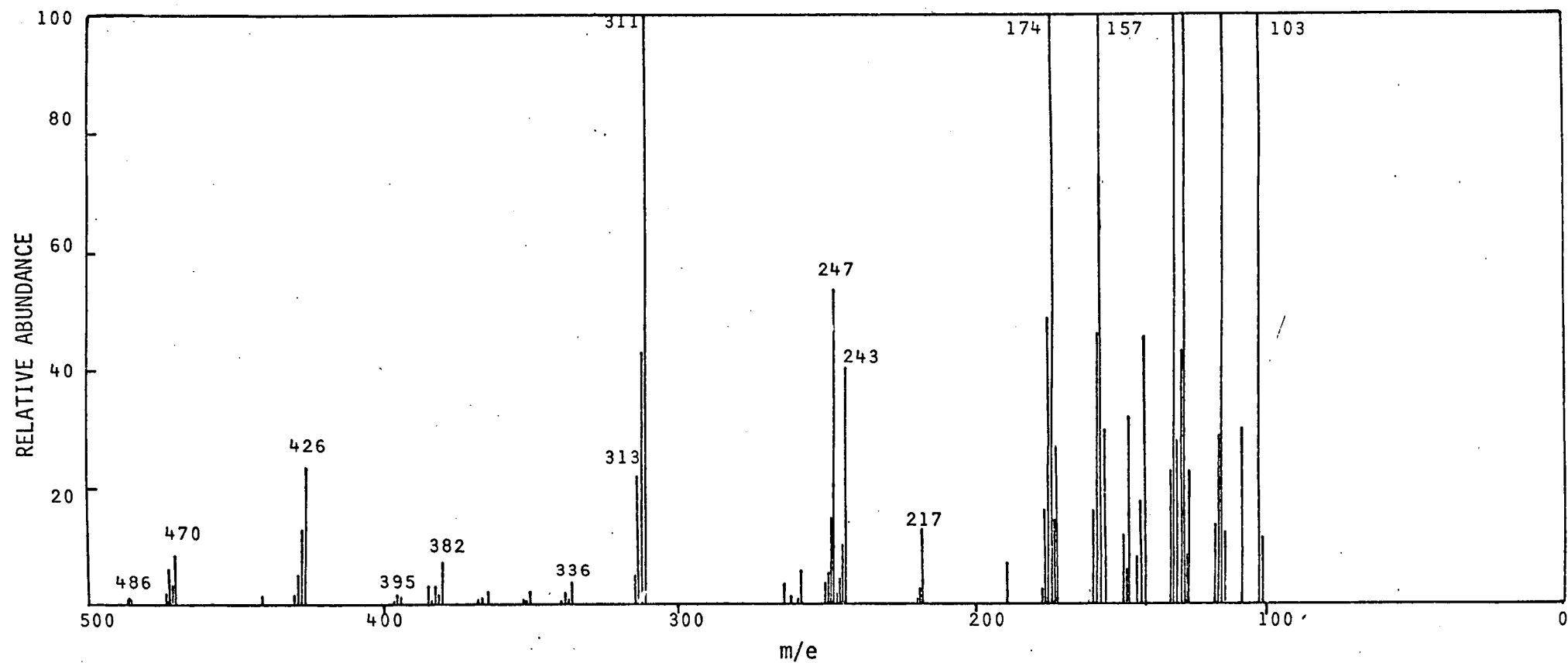
Fig. 3.   Partial mass spectrum of di-O-TMSi-N-acetyl-e-d18:1, derived from KL sphingenine sulphate.
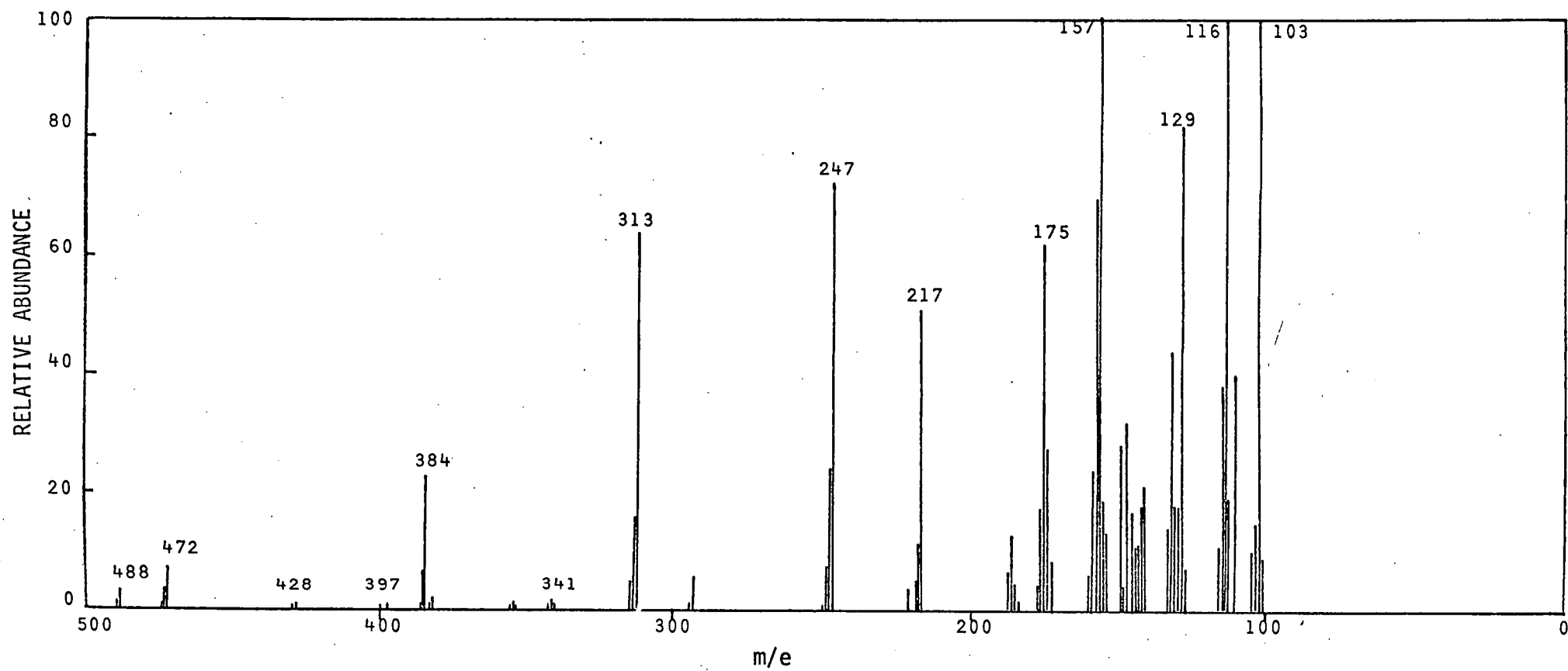
Fig. 4.   Partial mass spectrum of di-O-TMSi-N-acetyl-rac-d18:0, derived from KL rac-sphinganine.
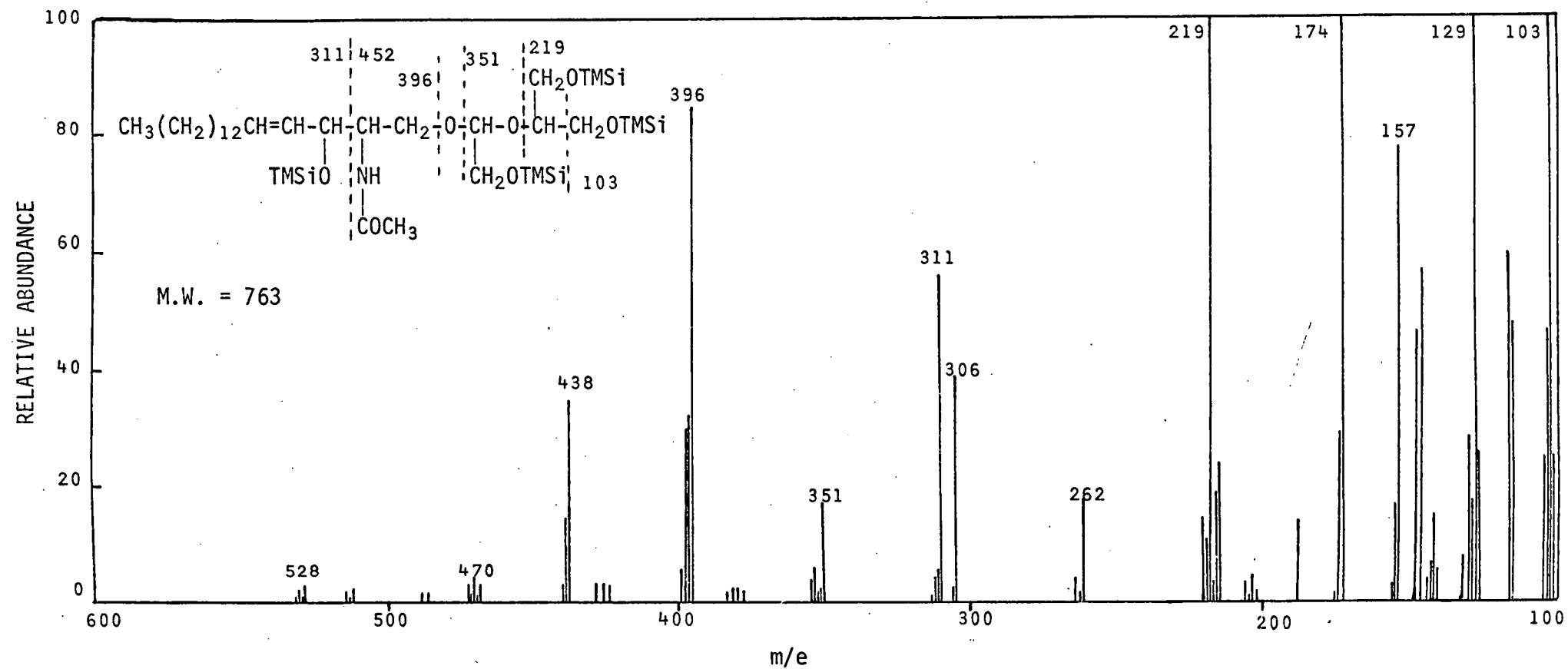
Fig. 5. Partial mass spectrum of the tetra-O-TMSi-N-acetyl sphingoids derived from KL "ceramide 3".

with losses of two trimethylsilyl radicals followed by a trimethyl-silanol molecule, respectively.

(d) Analysis of the sphingoids by GLC

The TMSi-N-acetyl sphingoids derived from the ceramides were analysed by the GLC method previously described (p. 36). "Ceramides 3 and 4" respectively were converted to "ceramides 1 and 2" before analysis. The two main sphingoids present in each ceramide were e-d18:1 and e-d18:0; these were present in the ratio 97:3 approximately. Other sphingoids occurred in traces and were ignored.

(e) Analysis of the fatty acids

The fatty acids derived from the ceramides after hydrolysis[93] were converted to methyl esters[98], and analysed by GLC as previously described (p. 36). The NFA derivatives ("ceramides 1 and 3") and HFA derivatives ("ceramides 2 and 4") respectively gave fatty acid compositions (Table 1) similar to those derived for cerebrosides (Table 2). The major fatty acids are, in decreasing order of importance, 24:1, 24:0, and 18:0 for NFA ceramides, and h24:0, h24:1, and h18:0 for HFA ceramides. For sphingomyelin (Table 2), 18:0, 24:1, and 24:0 are the major fatty acids, in decreasing order of importance.

The fatty acid compositions of bovine brain cerebrosides and sphingomyelin have been previously recorded, and are essentially the same as those observed here but for the absence of the longer chain fatty acids (C26 to C30)[99]. Such fatty acids have been found in human brain[100].

Table 1

Fatty acid composition of KL ceramides (weight %)

Fatty acid	"Ceramide 1"	"Ceramide 3"	"Ceramide 2"	"Ceramide 4"
12:0	0.2	trace	0.1	0.1
14:0	0.4	0.4	0.2	0.3
15:0	0.1	trace	-	0.2
16:0	5.0	3.9	1.1	0.1
16:1	-	-	trace	-
17:0	0.1	0.1	trace	-
18:0	11.9	10.7	15.3	14.6
18:1	0.4	0.3	0.6	0.2
18:2	1.6	0.2	-	-
19:0	trace	0.1	0.7	0.5
20:0	1.4	1.3	1.3	1.0
20:1	0.7	0.3	0.5	0.2
21:0	0.1	0.1	0.1	0.2
21:1	-	-	-	-
22:0	5.5	5.8	7.6	2.3
22:1	-	trace	-	-
23:0	5.8	5.8	8.4	7.3
23:1	0.2	0.2	0.3	-
24:0	21.3	20.0	28.1	31.0
24:1	32.6	31.1	16.1	16.2
25:0	2.2	2.3	4.0	3.5
25:1	1.3	0.9	1.8	0.5
26:0	3.1	3.3	5.5	4.2
26:1	5.1	3.5	5.3	1.0
27:0	0.2	4.3	0.7	6.9
27:1	0.4	0.1	trace	-
28:0	0.1	0.6	0.2	0.8
28:1	0.3	2.6	0.6	4.0
29:0	trace	1.5	0.5	2.6
29:1	-	0.3	trace	0.5
30:0	trace	1.9	0.3	1.5

Table 2

Fatty acid composition of bovine brain sphingolipids (weight %)

Fatty acid	Sphingomyelin	NFA cerebrosides	HFA cerebrosides
12:0	0.1	-	trace
14:0	0.1	trace	0.2
15:0	0.4	0.1	-
16:0	2.9	0.3	0.3
16:1	-	-	-
17:0	0.2	-	-
18:0	36.8	10.7	16.3
18:1	0.5	1.9	-
18:2	0.3	-	-
19:0	0.3	0.4	0.4
20:0	0.7	2.4	1.6
20:1	-	-	-
21:0	0.1	0.4	0.6
21:1	-	-	-
22:0	2.8	15.3	5.6
22:1	0.5	1.4	0.8
23:0	2.4	6.0	7.5
23:1	1.1	1.8	1.0
24:0	8.8	15.4	37.5
24:1	26.1	30.1	13.6
25:0	2.4	2.5	4.6
25:1	4.8	2.3	1.3
26:0	2.2	2.8	4.0
26:1	5.0	4.2	3.3
27:0	0.3	0.5	0.4
27:1	0.6	0.5	0.5
28:0	0.3	0.9	0.8
28:1	0.5	-	1.0
29:0	-	-	-
29:1	-	-	-
30:0	trace	-	-

2. Lipids accompanying natural ceramides

Other workers with serum ceramides[101] recorded relatively high amounts of C12 to C18 fatty acids, which are not usually found in such natural ceramides[102]. This suggested that contaminating fatty acids and monoglycerides have been overlooked. The relative chromatographic mobilities of these lipids, and their separation by special TLC techniques, have been described previously (p. 25). In TLC solvents containing chloroform and methanol, monoglycerides and HFA ceramides have similar chromatographic mobilities. However, only HFA ceramides were isolated by TLC from serum lipids[2]. Proof of the structures of the lipids which contaminate natural ceramides on TLC plates has been provided in the case of milk lipids; their isolation was carried out by preparative TLC.

(a) Identification and analysis of the fatty acids

The chromatographic mobility of the lipid fraction similar to that of NFA ceramides is ascribed to fatty acids. The IR spectrum of the fraction (Fig. 6) was practically identical to that of oleic acid, with absorption occurring at 3000 $\sim$ 2400 cm^{-1}, 1710 cm^{-1} and 1280 cm^{-1} for the carboxyl group, and 3000 cm^{-1} for the double bond. A weak band occurring at 1550 cm^{-1} could be an N-H deformation mode due to the presence of associated amino acids; ceramides are excluded by the absence of a C=O stretch at 1660 cm^{-1}, and by the fact that their concentration in milk is very small[103].

The fatty acids were converted to methyl esters[98], and analysed by GLC as previously described (p. 33). The composition was 18:1 (84%), 16:0 (14%), and 14:0 (2%); these are the major fatty acids of milk triglycerides.

(b) <u>Identification and analysis of the monoglycerides</u>

The chromatographic mobility of the lipid fraction similar to
that of HFA ceramides is ascribed to α-monoglycerides.   The IR
spectrum of the fraction (Fig. 7) was similar to that of synthetic
α-monopalmitin, with absorption occurring at 3360 cm^{-1} for ν O-H,
1740 cm^{-1} for ν C=O, 1150 cm^{-1} for the secondary ν C-O, and 1100 cm^{-1}
for the primary ν C-O.   The fraction gave positive colour tests for
ester[74] and vicinal diol[76] on the plate, and a negative colour test for
nitrogen[73].

The purity of the fraction was checked by carrying out colorimetric
analyses for glycerol[104], vicinal diol[105], and the number of ester
groups[106].   The values obtained respectively, per mg of lipid, were
2.30 mmole, 2.10 mmole, and 2.38 mmole.   The 1:1 correspondence
between these values gave an indication of α-monoglycerides, although
the slightly lower value for vicinal diol indicated the presence of
some β-monoglycerides which may have formed during TLC[107].   Pure
α-monoolein gave values of 2.80 mmole/mg, indicating that the fraction
was 85% pure;   the impurity was found to be mostly fatty acid.

3.   <u>Lipids derived from ceramides and related compounds</u>

The ageing of ceramides suggests oxidation and/or reacylation.
Of these two, the latter received more attention, as it occurred even
when precautions were taken against the former.

(a) <u>The stability of acyl sphingoids</u>

The non-reactivity of ceramides in chloroform-methanol (2:1)
solution, after standing for three weeks at room temperature, has been
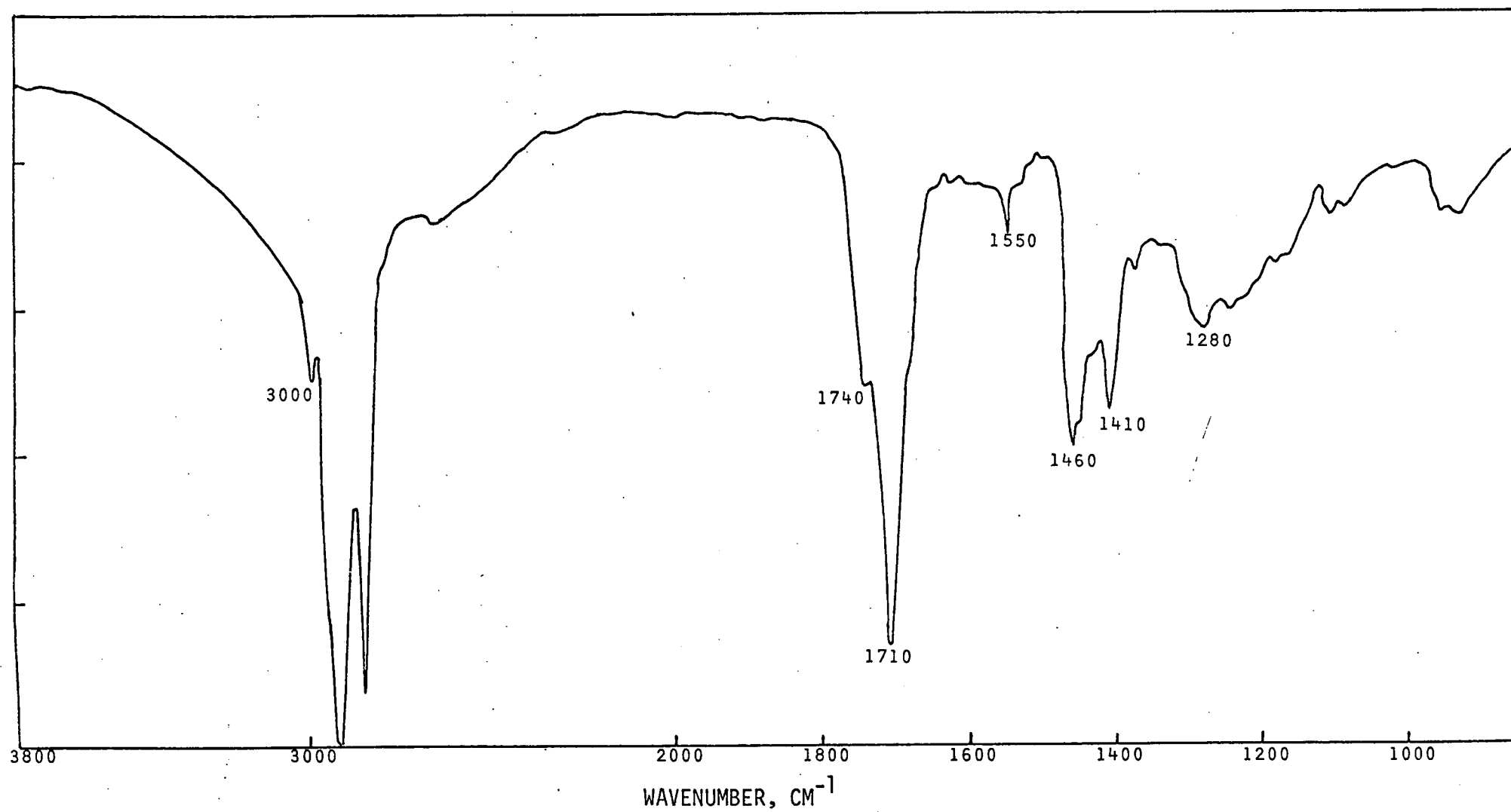
Fig. 6.   IR spectrum of free fatty acids derived from milk.
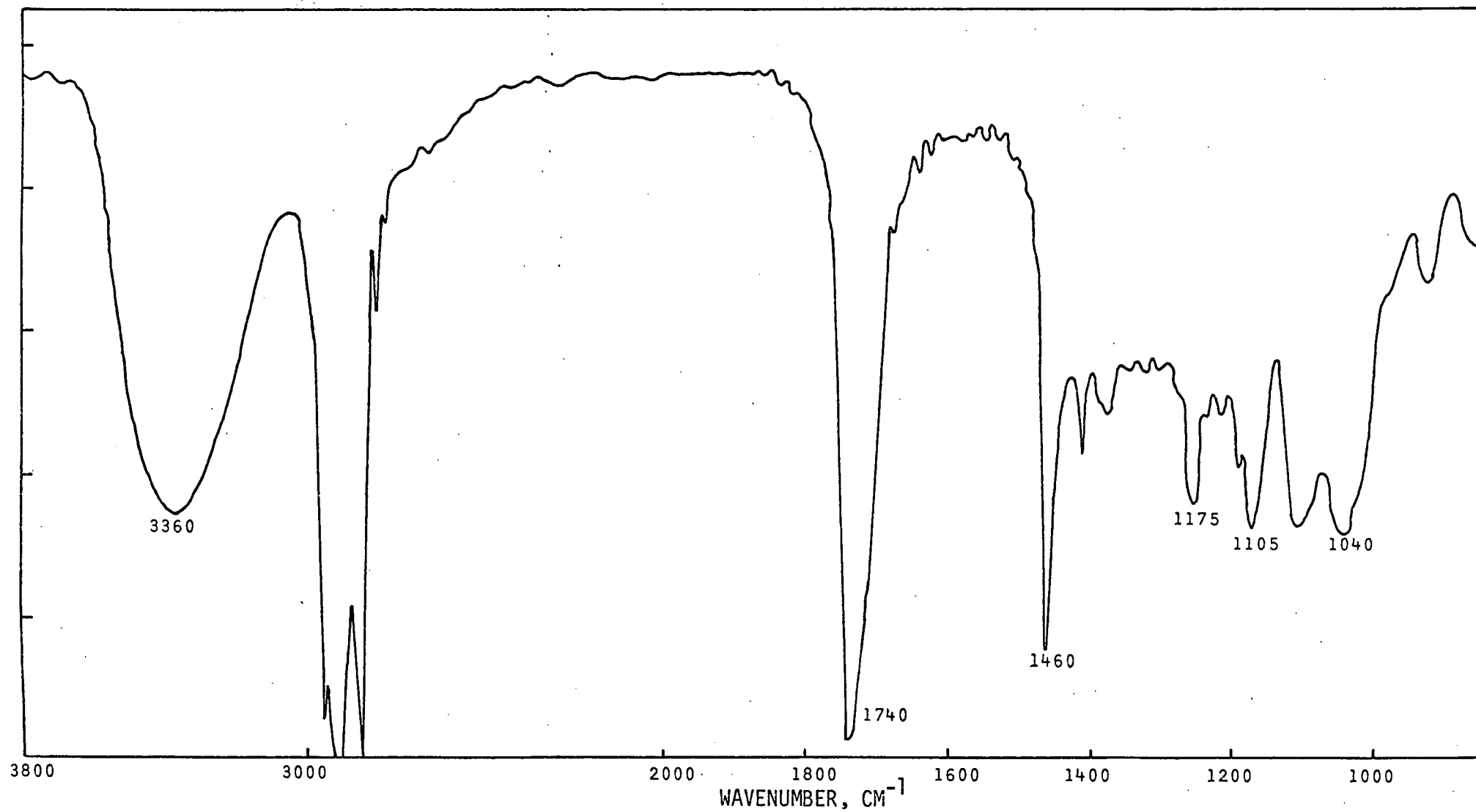
Fig. 7.   IR spectrum of monoglycerides derived from milk.

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noted[108]; during this time little or no change was detected on TLC plates. In this laboratory, similar experiments were conducted, with comparable results. Ceramides, stored either dry, in chloroform solution at 0°, or in chloroform solution at room temperature in the presence of sodium hydrogen carbonate or traces of sodium hydroxide, did not give any anomalous TLC spots during a period of three years. However, on TLC plates impregnated with borate, a minor component below the ceramides was observed when testing solutions which had been standing at room temperature for several months. As addition of ammonia or strong alkali to the solution did not remove the minor component, it was not an O-acyl sphingoid. Alkaline hydrolysis gave t-18:1, suggesting some inversion of the original ceramides. This may have been brought about by the action of a trace of hydrochloric acid formed in the ageing chloroform, although ceramide solutions containing added hydrochloric acid did not appreciably accelerate the process. The addition of hydrogen chloride to a dry ether solution of ceramides produced some O-acyl sphingoids, but unlike the reaction with N-palmitoyl-1-(p-nitrophenyl)-2-amino-1,3-propanediol[109], addition and hydrolysis products were also formed.

O-acyl sphingoids were stable when stored dry, but in chloroform solution at 0° isomerisation to the N-acyl compounds was complete after several weeks.

(b) Natural ceramides and related compounds

Ceramides have been found in nearly every type of tissue of animal origin, and in most cases, the relative concentration is small. Hence the study of compounds related to ceramides in lipids from plasma, erythrocytes, liver, kidney, brain, spleen, aorta, and lung, was found to be impractical. Platelet lipids, which are rich in

ceramides (0.5 to 1.3% ^{W}/w)[110,111] and relatively poor in neutral

lipids, were potentially the most practical source. Platelet

ceramides also have a high turnover rate[112].

The platelets obtained from ox blood (5 ℓ)[110] were extracted[38],

and the lipids tested for ceramides and related compounds by TLC

(Fig. 8). As observed (lane 2), NFA ceramides are resolved into

three components (probably due to differences in the fatty acid moeity),

while compounds corresponding to HFA ceramides appear as two components.

The results obtained by adding ammonia to the lipid solution were not

conclusive in suggesting the presence of small amounts of O-acyl

sphingoids in platelets, since the TLC plates appeared much the same

before and after treatment (except for acidic components). The two

components corresponding to HFA ceramides were respectively found to

be monoglycerides, and possibly HFA ceramides, by 2-dimensional

chromatography (p. 26); the latter has not been observed before in

platelets.

On other TLC plates, sphingoid-oxazolines were not found in

platelet lipids after the removal of interfering glycerides with mild

alkali[45].

(c) Starting materials for oxazolines

The preparation of oxazolines from sphingoids, acyl sphingoids or

related compounds was attempted by three methods:

i) dehydration of N-acyl derivatives;

ii) condensation of β-hydroxyamino acid esters with imidic

acid ester hydrochlorides;

iii) condensation of sphingoids with imidic acid ester

hydrochlorides.
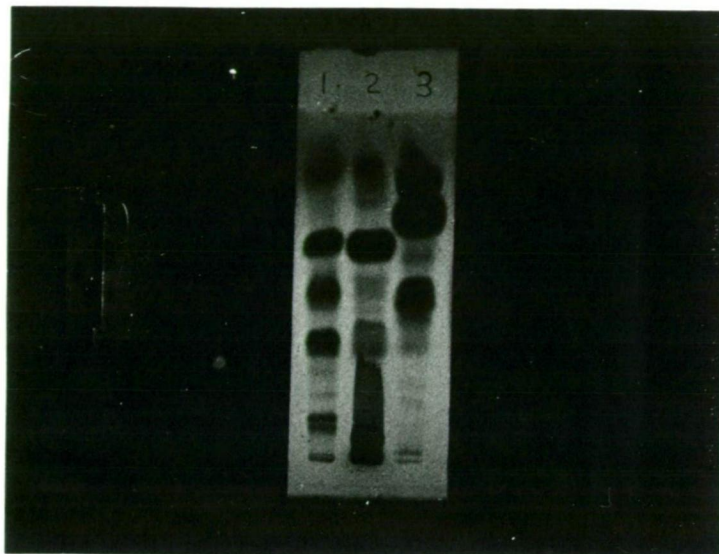
Fig. 8.   TLC plate illustrating ceramides from ox platelets.
Developing solvent, chloroform-carbon tetrachloride-methanol-water
(50:50:12:0.5).   1, cholesterol (Rf 0.64), ceramides (Rf 0.50, 0.35),
and cerebrosides (Rf 0.12, 0.09); 2, platelet lipids; 3, sphingoid-
oxazolines (Rf 0.80, 0.70) and acyl sphingoids (Rf 0.50, 0.45).

i) Dehydration of ceramides and related compounds was attempted with thionyl chloride. Of the compounds tested, only threonine and allothreonine derivatives containing long-chain fatty acyl groups formed oxazolines without side reactions (N-phenyl analogues gave similar results[113]). Ceramides and N-acyl phenyl-serinols gave β-chloro substituted oxazolines; phenylserine derivatives produced no oxazoline at all, but only β-chloroamides. From these results, use of thionyl chloride as a dehydrating agent would appear unsuitable for the preparation of oxazolines from amides with a β-hydroxy group in the benzylic or allylic positions[114,115].

ii) The condensation of β-hydroxyamino acid esters with either aliphatic or aromatic imidic acid ester hydrochlorides gave oxazolines in quantitative yields. The reaction using free imidic acid esters and β-hydroxyamino acid ester hydrochlorides gives relatively low yields[116], and was not used. Reduction of the ester group to the alcohol could be carried out successfully with lithium aluminium hydride; the double bond of the ring was not affected under the conditions used, although 2-alkyl oxazolines of a similar nature have been stated to be susceptible to attack by this reducing agent.

The preparation of long chain alkyl β-hydroxyamino acid analogues would be advantageous because of the high yields of oxazolines, and the unambiguous nature of their synthesis. Also, the configuration of the oxazolines may be determined by using Elliot's observation that sodium ethoxide catalyses the transformation of <u>cis</u>- to <u>trans</u>-oxazolines[116,117]. The preparation of the isomers of α-amino-β-hydroxystearic acid by methods other than existing ones[118,119], was attempted. Copper II-Schiff base chelates have been successfully condensed with short chain aldehydes under base-catalysed conditions, producing β-hydroxyamino acids in yields usually greater than 60%[120,121].

The reactions were carried out in either water or methanol in which the reactants were soluble. In the present work, yields of the derivatives were found to be low, owing to the insolubility of the aldehydes in the reaction media.

iii) Sphingoids were found to condense with imidic acid ester hydrochlorides in good yield (60-80%). The presence of the three adjacent functional groups available for reaction led to a mixture of oxazolines which required methods for their separation and characterisation. These aspects are discussed in the next section.

(d) The chemistry of sphingoid-oxazolines

The reaction scheme (Fig. 9) for the preparation and degradation of sphingoid-oxazolines illustrates four isomeric compounds (I), (II), (III), and (IV) which may be derived from the condensation of e- and t-sphingoids with imidic acid ester hydrochlorides. It was found that (I) and (III) were the major products of the synthesis, and (II) and (IV) the minor products. Solutions of the oxazolines displayed an equilibrium in which the more stable isomers were favoured (see Ch. 3, p. 91).

Purification of a mixture of the oxazolines could be carried out by recrystallization from ethanol, or by preparative TLC followed by recrystallization of the oxazoline (I).

Under mildly acidic conditions[122], the oxazolines (I) and (III) were unstable, yielding the acid salts of the O-acyl sphingoids (V) and (VI), respectively. On careful treatment with ammonia to pH 5-6[116], the free O-acyl sphingoids (VII) and (VIII) were obtained.
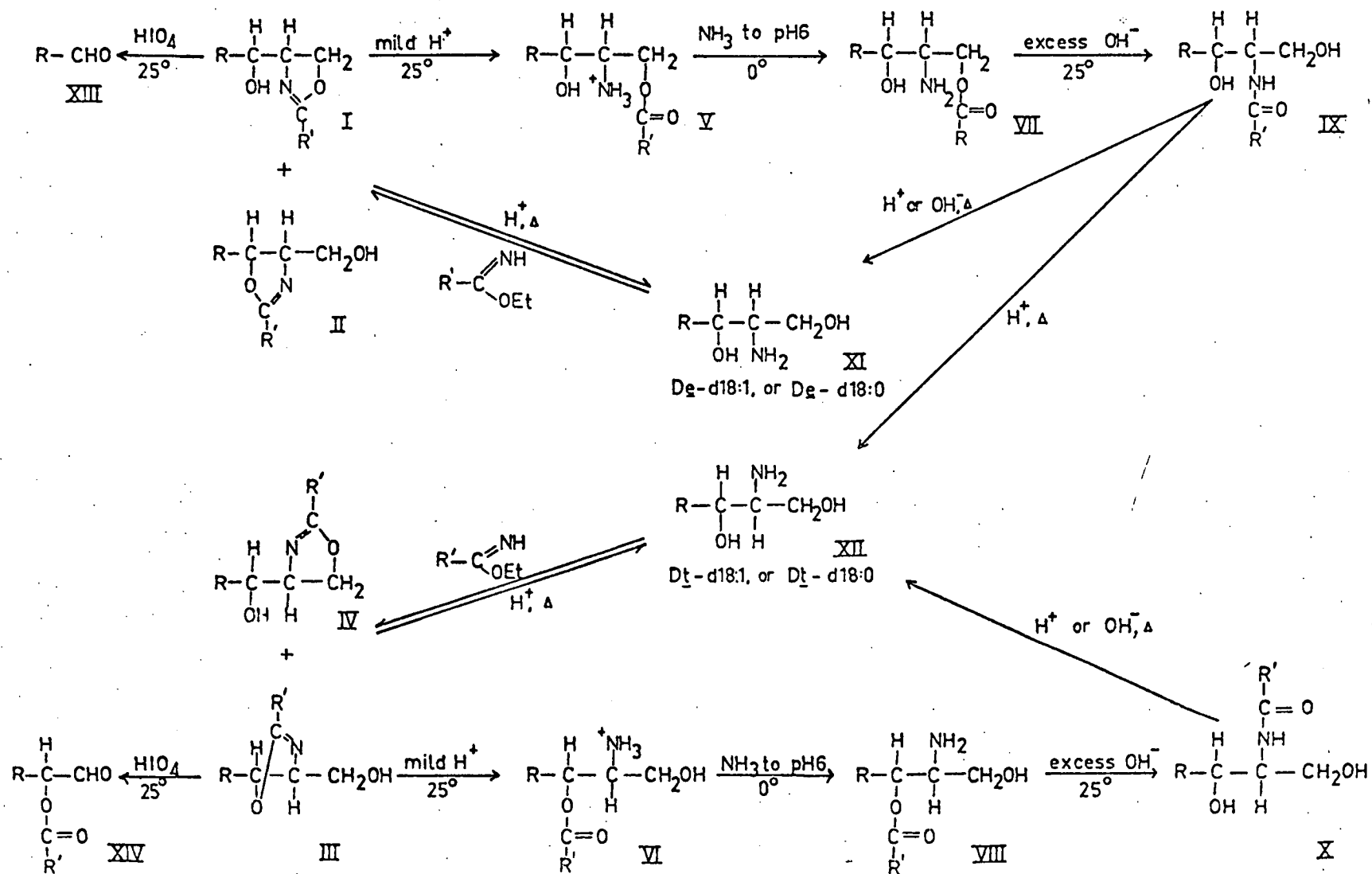
Fig. 9.   Reaction scheme for the preparation and degradation of sphingoid-oxazolines.

The addition of more ammonia to pH 8 or greater caused the acyl group to migrate, and the ceramides (IX) and (X) were isolated without change of configuration.

The position of the free hydroxyl group in the oxazolines (I, II, III, or IV) may be verified by examining the periodate oxidation products[123]. Ring cleavage and oxidation were carried out with periodic acid in pyridine[124]. The aldehydes liberated, and those liberated from either d18:0 or d18:1 were compared on TLC plates (developing solvent, hexane-diethyl ether (9:1))[125]. Hexadecan-1-al (R_f, 0.50) and trans-2-hexadecen-1-al (R_f, 0.41) were produced from d18:0 and d18:1 respectively, and only the oxazolines (I) and (IV) gave these aldehydes (XIII). The oxidation products of the oxazolines (III) and (II) did not show TLC spots corresponding to these aldehydes, and presumably the compounds (XIV) were produced.

The spectroscopic, chromatographic, and stereochemical differences in the products illustrated in Fig. 9 are treated in Chapter 3.

(e) Oxidation of ceramides

Unsaturated lipids can be susceptible to photooxidation and autoxidation under normal storage conditions, the latter can be prevented by storing lipids either dry or in solution, under nitrogen, and by using antioxidants. During TLC, lipids are quite stable to autoxidation[126].

The photooxidation of lipids has not been critically investigated. Hence, experiments were conducted to assess the effects of various conditions, especially those under which one works normally in the laboratory, on lipid residues. It was found that unsaturated lipids exposed to natural light gave reaction products of slower chromato- graphic mobility than the parent lipids; the reactivity increased as

the degree of unsaturation increased. The photooxidation of ceramides, and some common neutral lipids, are illustrated in Fig. 10 and Fig. 11, respectively. The parent lipids had been applied to the origin of the TLC plate, and exposed to subdued sunlight for one day; similar results were obtained for lipids finely dispersed on a watch-glass. Negative results were obtained for saturated lipids, or lipids stored in the absence of sunlight under normal laboratory conditions (or in pure oxygen, either dry or saturated).

4. Experimental

(a) Attempted dehydration of compounds related to ceramides

(i) Phenylserine

Benzaldehyde (400 g) was added with stirring to glycine (150 g) and sodium hydroxide (120 g) in water (500 ml) at 5-10° and allowed to stand overnight. The crude product was macerated, treated with concentrated hydrochloric acid (250 ml), cooled in ice overnight, filtered, and recrystallized from aqueous ethanol. The yield was 260 g (71%), and its m.p. was 194-5° as reported[127].

(ii) Phenylserine ethyl ester

Hydrogen chloride was bubbled for several hours through a suspension of phenylserine in dry ethanol. The solution was concentrated under reduced pressure yielding the hydrochloride salt of phenylserine ethyl ester (m.p. 132° after washing with dry ether). This was dissolved in ice-cold water, excess potassium carbonate added, and the free ester extracted with ether. The product, recrystallized twice from ether, melted at 82°. On another occasion the same sequence of steps gave a product which melted at 87°. Both products were pure, as indicated by their homogeneity on TLC plates, NMR spectroscopy, and
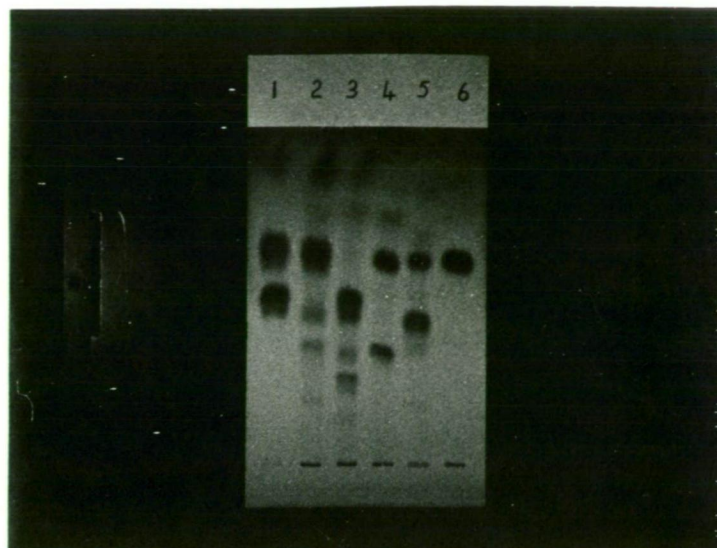
Fig. 10.   TLC plate illustrating some photooxidation products of ceramides resulting from exposure to subdued natural light for one day (lanes 2-6).   Developing solvent, chloroform-carbon tetrachloride-methanol-water (50:50:12:0.5).   1, KL ceramides (freshly applied); 2, NFA ceramides, natural mixture; 3, HFA ceramides, natural mixture; 4, 24:0-De-d18:1; 5, 24:1-De-d18:0; 6, 24:0-De-d18:0.
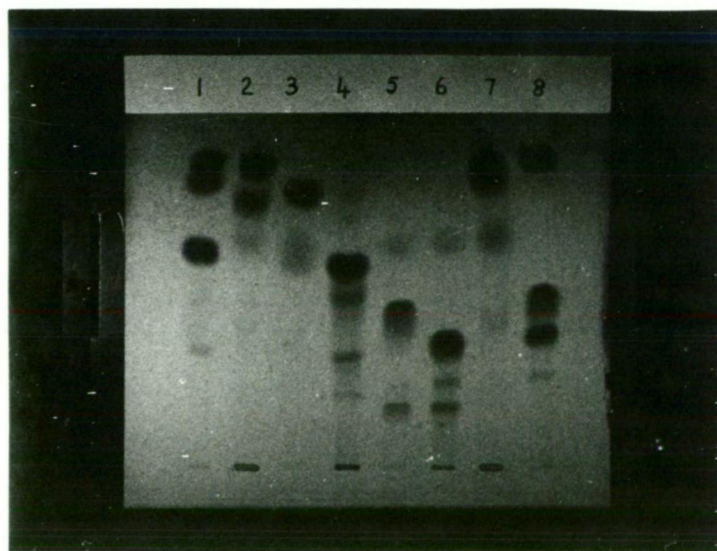
Fig. 11.   TLC plate illustrating some photooxidation products of neutral lipids (lanes 2-7).   Developing solvent, chloroform-carbon tetrachloride-methanol-water (50:50:12:0.5).   1, cholesteryl oleate (Rf 0.80), methyl oleate (Rf 0.73), and cholesterol (Rf 0.57), freshly applied; 2, cholesteryl oleate; 3, methyl oleate; 4, cholesterol; 5, oleic acid; 6, monoolein; 7, triolein; 8, triolein (Rf 0.80), oleic acid (Rf 0.45), and monoolein (Rf 0.35), freshly applied.

elemental analysis. The literature gives a melting point of 138°, and assumes that it is the t-isomer[127]. The results obtained here indicate that its diastereoisomer may also be present (Analysis: C, 63.1; H, 7.2; N, 6.8%. Calcd. for $C_{11}H_{15}O_3N$: C, 63.1; H, 7.2; N, 6.7%).

(iii) DLt-phenylserinol (DLt-1-phenyl-2-amino-1,3-propanediol)

Phenylserine ethyl ester (10 g) in dry ether (400 ml) was treated with lithium aluminium hydride. The viscous liquid obtained was recrystallized from ether to give t-phenylserinol, m.p. 88° (as reported[127]), in 30% yield (2.4 g)(Analysis: C, 64.7; H, 7.8; N, 8.3%. Calcd. for $C_9H_{13}O_2N$: C, 64.7; H, 7.8; N, 8.4%). The remainder, consisting most likely of e-phenylserinol, could not be induced to crystallize.

(iv) N-stearoyl phenylserine ethyl ester; reaction with $SOCl_2$

Stearoyl chloride[128] (0.01 mole, or 3.03 g) in N,N-dimethylformamide (10 ml) was added slowly to a solution of phenylserine ethyl ester (0.01 mole, or 2.093 g) in N,N-dimethylformamide (30 ml) and triethylamine (2 ml), and the solution stirred for two hours at room temperature. The reaction mixture was then added to an ice-water mixture (200 ml), acidified with dilute hydrochloric acid, and the crude product filtered and washed thoroughly with water. Recrystallization from petroleum ether (60-70) yielded 4.37 g (95%) of N-stearoyl phenylserine ethyl ester, m.p. 55-56°.

Thionyl chloride (4 ml) was added dropwise to a solution of N-stearoyl phenylserine ethyl ester (3.325 g) in chloroform (4 ml) at 0° and left to stand overnight. The solvents were removed under reduced pressure at room temperature, the residue dissolved in diethyl ether, washed with sodium carbonate solution, and the solvent removed

to yield 3.3 g of material. This was applied to a column of silicic acid (100 g), and ethyl-(N-stearoyl-3-phenyl-3-chloro-2-amino) propanoate eluted with chloroform in 70-80% yield (m.p. 75-76°).

(v) N-stearoyl t-phenylserinol; reaction with SOCl$_2$

The reaction conditions for the preparation of N-stearoyl t-phenylserinol were the same as those for the preparation of N-stearoyl phenylserine ethyl ester. The product was obtained in 79% yield, and when recrystallized from methanol melted at 78-80° (the p-nitro analogue melts at 92-94°[129]).

The products resulting from the reaction of this compound with thionyl chloride at 0° were numerous. IR spectroscopy identified some of the minor products as amides and esters containing a chlorine group on the carbon atom in the α-position to the phenyl group. The major product, isolated in 40-50% yield by silicic acid column chromatography, was 2-heptadecyl-4-(1-phenyl-1-chloromethyl)-2-oxazoline. This compound was non-reactive towards silver carbonate and hot sodium hydroxide solution, remained a colourless oil at 0°, and showed strong absorption at 1665 cm^{-1} (ν C=N) in the IR spectrum. The NMR signals were clearly resolved, occurring at 2.75 τ (for 5 aromatic protons), 3.1 τ (for 1 proton on the carbon containing the Cl), 3.8 τ (for 2 protons in the 5-position on the ring), 7.85 τ (for 1 proton in the 4-position on the ring), and 8.75 and 9.1 τ (for the aliphatic protons).

(vi) N-isonicotinoyl phenylserinol

Isonicotinoyl chloride hydrochloride was prepared by heating a solution of isonicotinic acid in thionyl chloride at 60° for 1 hour[130]. The solvent was removed under reduced pressure, and the residue sublimed onto a cold finger at 1 mm Hg pressure. It melted at 157-160° (dec.);

after one recrystallization the m.p. rose to 164-165°, as reported[130].

Isonicotinoyl chloride hydrochloride (1.066 g, or 6 mmole) in N,N-dimethylformamide (20 ml) was added with stirring to $\underline{t}$-phenylserinol (1,000 g, or 6 mmole) in N,N-dimethylformamide (20 ml) and triethylamine (2 ml). After standing overnight the triethylamine hydrochloride was removed by filtration, the solvent removed under reduced pressure, and the residue dissolved in hot chloroform. The free isonicotinic acid was removed by filtration, and the chloroform cooled to yield 1.55 g (95%) of N-isonicotinoyl $\underline{t}$-phenylserinol, m.p. 145-146° (white flakes). Recrystallization from 2% ethanol in chloroform yielded the same compound, which gave one spot on TLC plates (Analysis: C, 65.6; H, 5.9; N, 10.1%. Calcd. for $C_{15}H_{16}O_3N_2$: C, 66.1; H, 5.9; N, 10.3%).

The compound was soluble in water and alcohols, sparingly soluble in hot chloroform, and insoluble in ether and hydrocarbons. Its IR spectrum showed absorption at 3390 cm^{-1} (ν OH), 3300 cm^{-1} (ν NH), 3230 cm^{-1} and 1630 cm^{-1} (ν C=O), and 1540 cm^{-1} (δ NH). The UV spectrum gave peaks at 211 mμ (ε max = 18100) and 252 mμ (ε max = 7200) for the pyridyl and phenyl groups.

(vii) Reaction of a natural ceramide mixture with $SOCl_2$

The action of thionyl chloride at 0° on NFA ceramides produced a mixture of β-chloroamides, β-chlorooxazolines (ν C=N, 1660 cm^{-1}), and other products.

(b) Long-chain β-hydroxyamino acids

(i) Copper II-Schiff base complexes

N-salicylideneglycinataquo copper II dihydrate was prepared from salicylaldehyde, glycine, and cupric acetate, as described[131]. After

recrystallization from water-ethanol mixtures, dark green crystals were obtained.

N-pyruvylideneglycinatoaquo copper II dihydrate was prepared essentially as described[120], with the exception that the final product was recrystallized from water.   It decomposed at 206° (reported, 195°).

N-pyruvylideneglycinatopyridine copper II trihydrate was prepared by treating the above compound with pyridine in dry acetone[120].   The product decomposed at 142° (reported, 133°).

(ii) α-amino-β-hydroxystearic acid

The preparation of a long-chain β-hydroxyamino acid proved to be difficult owing to the insolubility of the aldehyde in the aqueous-methanolic media necessary to dissolve the complex.   Several variations of the method described here were tried, but yields were poor (5% or less).

To a mixture of N-pyruvylideneglycinatoaquo copper II dihydrate (1.3 g, or 5 mmole) and hexadecanal[132]   (2.4 g, or 10 mmole) in methanol (40 ml) and diethyl ether (5 ml), were added 1 N sodium methoxide (7 ml).   The mixture was stirred under nitrogen overnight at 35°.   After this time, 3 N acetic acid (5 ml), water (50 ml), and diethyl ether (100 ml) were added, and the products partitioned into the ether.   Evidence for the presence of α-amino-β-hydroxystearic acid was found in its IR spectrum, its positive reaction with ninhydrin and the chromatographic behaviour of its DNP derivative.   The latter experiment indicated the presence of e- and t-isomers, since two spots were found on TLC plates impregnated with borate (R_f values, 0.63 and 0.75; developing solvent, chloroform-methanol-water (65:25:4)).

An equally unsuccessful attempt to prepare the above compound was made using N-pyruvylideneglycinatopyridine copper II trihydrate.

This complex was found to be more soluble in the methanol-ether mixtures used for the reaction, but yields were still low owing to the insolubility of the aldehyde.

(iii) α-amino-β-hydroxy-<u>trans</u>-4-hexenoic acid

N-pyruvylideneglycinatoaquo copper II dihydrate (2.6 g, 10 mmole) and crotonaldehyde (2.1 g, 30 mmole) were dissolved in water (50 ml), and sodium hydroxide added to bring the pH up to 9.5. The solution was stirred at room temperature for four hours, after which the pH was adjusted to 4.5 with 3 N acetic acid. It was then poured onto a column (3 cm x 10 cm) of Amberlite IR-120 (H form). The column was washed with distilled water (1 ℓ), and the amino acids eluted with 2 N aqueous ammonia (1 ℓ). The solvent was removed to produce a stereoisomeric mixture of DL-α-amino-β-hydroxy-<u>trans</u>-4-hexenoic acids (impure), m.p. 200° (dec.). The mixture had similar chromatographic properties as the DL-α-amino-β-hydroxy-4-hexynoic acids[133].

(c) Oxazolines and their degradation products from β-hydroxyamino acids

Phenylserine ethyl ester, or threonine ethyl ester[134], were condensed with aliphatic or aromatic imidic acid ester hydrochlorides to yield oxazolines. Their lithium aluminium hydride reduction products gave O-acyl derivatives on mild hydrolysis; further treatment with alkali afforded N-acyl derivatives. The accompanying tables (Tables 3,4) give some properties of the compounds prepared.

(i) Imidic acid ester hydrochlorides[135]

Lauronitrile (18.1 g, or 0.1 mmole) and absolute ethanol (4.5 g, or 0.1 mole) were treated with hydrogen chloride (4.4 g, or 0.12 mole)

at room temperature for three hours. Dry ether (100 ml) was added, and the mixture left at 0° overnight to yield 18 g of ethyliminolaurate hydrochloride, m.p. 79-80° (dec.). Similarly prepared were ethylimino-myristate hydrochloride, m.p. 82-84° (dec.), ethyliminostearate hydrochloride, m.p. 87-89° (dec.), and ethyliminobenzoate hydrochloride, m.p. 114-115° (dec.); the latter was prepared at 0°.

(ii) 2,5-dialkyl-4-carboethoxy-2-oxazolines

The following method is typical. Phenylserine ethyl ester (0.02 mole) and ethyliminolaurate hydrochloride (0.02 mole) were dissolved in anhydrous chloroform (50 ml) and refluxed for one hour. The ammonium chloride was washed out with water, or removed by centrifuging at 15000 r.p.m., and the chloroform removed at 40° under reduced pressure. The product was used without purification for reduction of the carboethoxy group, as follows.

(iii) 2,5-dialkyl-4-hydroxymethyl-2-oxazolines

A stirred suspension of lithium aluminium hydride (0.6 g) in dry ether (50 ml) was boiled for 10 min and cooled in ice. To this was added, dropwise, a solution of an ester (0.015 mole) in dry ether (50 ml). The mixture was stirred for a further 10 minutes at room temperature, refluxed for 10 minutes, cooled, and the excess reagent hydrolysed by adding water (1 ml) and 10% sodium hydroxide (2 ml) dropwise. The lithium and aluminium salts were removed by filtration, and the ether removed under reduced pressure. The residual oil was dissolved in hot ether or hexane, filtered, cooled in a mixture of ice and salt, and the crystals collected.

(iv) 1-0-acyl-phenylserinol hydrochlorides

A solution of the oxazoline from (iii) in 1 N methanolic hydrochloric acid was left at room temperature overnight. The solvent was removed at room temperature under reduced pressure, and the residue recrystallized from an appropriate solvent.

(v) N-acyl-phenylserinols

A solution of the 0-acyl derivative from (iv) in methanol was treated with dilute ammonia carefully to pH 5-6. Chloroform was added, the ammonium chloride washed out with water, and the solvent removed at room temperature under reduced pressure to yield the N-acyl derivatives; recrystallization from chloroform-hexane gave a pure product. The free 1-0-acyl phenylserinols were not stable enough for their isolation under these conditions; 0-benzoyl threonines have been isolated, however[116]. Storage of the hydrochlorides over potassium hydroxide in a vacuum did not liberate the free 0-acyl derivatives.

(vi) Configuration of the oxazolines[117]

2-Undecyl-5-phenyl-4-carboethoxy-2-oxazoline (1.50 g) was hydrolysed to the 0-acyl derivative by treatment for 18 hours at room temperature with 0.5 N methanolic hydrochloric acid (25 ml). The solution was neutralized with 2 N sodium hydroxide, a further 5 ml of this reagent added, and refluxed for 30 minutes. After cooling, 2 N hydrochloric acid (10 ml) and ice-water (100 ml) were added, the precipitate collected, dried over KOH, and recrystallized twice from chloroform-hexane (1:6). N-undecanoyl phenylserine was obtained in 90% yield (1.31 g), m.p. 112-113°. The IR spectrum showed absorption

at 2310 cm^{-1} (ν N-H), 1700 cm^{-1}, 1725 cm^{-1} (ν C=O for the acid),

1643 cm^{-1} (ν C=O for the amide), and 1523 cm^{-1} (δ N-H). Analysis by

TLC on plates impregnated with borate revealed the presence of a

single compound (developing solvent, chloroform-methanol-water

(65:25:4); R$_f$ value, 0.60).

2-Undecyl-5-phenyl-4-carboethoxy-2-oxazoline (1.50 g) was treated

with sodium ethoxide (2.5 g) in dry ethanol (25 ml). After 10 minutes

water (5 ml) was added, the solution refluxed for 15 minutes, then

acidified with hydrochloric acid to 0.5 N and allowed to stand overnight

at room temperature. The N-acyl compound was formed by adding 10%

sodium hydroxide to pH 11, and acidifying with dilute hydrochloric acid.

Recrystallization from chloroform-hexane (1:6) afforded crystals in

85% yield (1.24 g), m.p. 112-113°. Its IR spectrum and chromatographic

properties were identical to those of the product obtained from direct

acid hydrolysis, indicating the <u>trans</u>-configuration for the oxazolines.

(d) <u>Oxazolines and their degradation products from sphingoids</u>

Sphingoids were condensed with aliphatic imidic acid ester

hydrochlorides to yield oxazolines. A reaction scheme illustrating

some of their degradation products has been described (Fig. 9, p. 59).

All compounds prepared are tabulated (Tables 3,4).

(i) <u>Oxazolines</u>

A solution of a sphingoid (1 mmole, or 300 mg) and imidic acid

ester hydrochloride (1 mmole) in dry chloroform (4 ml) was refluxed

for 3 hours under nitrogen, cooled, and the liberated ammonium

chloride washed out with water. The chloroform was removed under

reduced pressure at room temperature, and the oxazoline purified

either by recrystallization from ethanol, or by preparative TLC

(developing solvent, 2% CH_3OH in $CHCl_3$). In general the yields were good (60-80%), and fine white crystals were obtained from pure starting materials.

(ii) O-acyl sphingoids

A solution of the sphingoid-oxazoline (0.4 mmole) in chloroform (10 ml) was acidified with 0.1 N sulphuric acid (0.22 mmole), and allowed to stand for three days at room temperature under nitrogen. After this time, the excess acid was washed out by adding methanol (2.8 ml) and water (4 ml), and shaking. The lower chloroform layer was withdrawn, evaporated to dryness at room temperature under reduced pressure, and the residue recrystallized from methanol to yield O-acyl sphingoid sulphates.

The acid salt (0.25 mmole) in chloroform (10 ml) was treated with 0.05 N methanolic ammonia (5.2 ml, or 0.26 mmole) at 0°. Water (4 ml) was added, and the free esters partitioned into the chloroform. The solvent was removed at room temperature under reduced pressure, and the ester recrystallized from methanol.

(iii) N-acyl sphingoids

The O-acyl isomers in chloroform were treated with methanolic sodium hydroxide. After acidifying and washing out the salt and free acid, the residue was purified by TLC, and recrystallized from methanol or ethanol to yield pure ceramides.

Table 3

Oxazolines derived from phenylserine, threonine, or sphingoids[a]
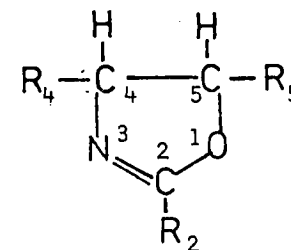
No.	Oxazoline substituents R_2	R_4	R_5	M.p.°	Yield %	IR data (Nujol)	Formulae	C % Found (Calcd.)	H % Found (Calcd.)	N % Found (Calcd.)
1	Und	cEy	Ph	10-11	100	1660 (ν C=N) 1735 (ν C=O)	$C_{23}H_{35}O_3N$	73.7 (73.9)	9.8 (9.4)	4.1 (3.8)
2[c]	Heptad	cEy	Ph	42-43	100	1660 (ν C=N)				
3[b,c]	Ph	cEy	Ph	<0	100	1642 (ν C=N) 1735 (ν C=O)				
4[c]	Und	cEy	Me	-7	100	1660 (ν C=N) 1740 (ν C=O)				
5[b,c]	Ph	cEy	Me	<0	100	1645 (ν C=N)				
6[c]	Heptad	cEy	Me	28	100	1660 (ν C=N)				
7	Und	hMe	Ph	49.5	85	1660 (ν C=N) 3230 (ν O-H)	$C_{21}H_{23}O_2N$	76.2 (76.1)	10.0 (10.0)	4.1 (4.2)
8	Ph	hMe	Ph	160	82	1644 (ν C=N) 3155 (ν O-H)	$C_{16}H_{15}O_2N$	76.1 (75.9)	6.0 (6.0)	5.6 (5.5)

Table 3 contd.

9	Und	hMe	Me	42.5	82	1660 (ν C=N) 3250 (ν O-H)	$C_{16}H_{31}O_2N$	71.3 (71.3)	11.4 (11.6)	5.3 (5.2)
10	Ph	hMe	Ph	87	86	1642 (ν C=N) 3170 (ν O-H)	$C_{10}H_{13}O_2N$	69.0 (69.1)	7.0 (6.9)	7.6 (7.3)
11	Trid	1h-Hexad$^{\Delta 2}$	H	89-90	68	1665 (ν C=N) 3150 (ν O-H)	$C_{32}H_{61}O_2N$	78.6 (78.2)	12.3 (12.5)	2.6 (2.8)
12	Trid	1h-Hexad	H	74-75	79	1665 (ν C=N) 3230 (ν O-H)	$C_{32}H_{63}O_2N$	77.2 (77.8)	12.8 (12.9)	2.6 (2.8)
13	Trid	hMe	Pentad	47-49	74	1655 (ν C=N) 3245 (ν O-H)	$C_{32}H_{63}O_2N$	77.7 (77.8)	12.8 (12.9)	2.7 (2.8)

a) All compounds are new.   b) could not be induced to crystallize.   c) have not been sent for microanalysis.

Table 4

Esters and amides derived from phenylserine and sphingoids[a]

No.	Compound Name	M.p.°	Yield %	IR data (Nujol)	Formulae	C % Found (Calcd.)	H % Found (Calcd.)	N % Found (Calcd.)
1[b]	1-0-undecanoyl phenylserinol, HCl salt	134-5 (dec.)	92	1730 (ν C=O) 3360 (ν O-H)	$C_{21}H_{36}O_3NCl$	65.3 (65.4)	9.4 (9.4)	3.9 (3.6)
2[c]	1-0-benzoyl phenylserinol, HCl salt	177-9 (dec.)	95	1725 (ν C=O) 3400 (ν O-H)	$C_{16}H_{18}O_3NCl$	62.1 (62.4)	5.9 (5.9)	4.5 (4.6)
3	N-undecanoyl phenylserinol	96-97	100	1635 (ν C=O) 1525 (δ N-H)	$C_{21}H_{35}O_3N$	72.2 (72.5)	10.1 (10.2)	4.0 (3.9)
4[d]	N-benzoyl phenylserinol	161.5	93	1625 (ν C=O) 1545 (δ N-H)	$C_{16}H_{17}O_3N$	69.4 (70.8)	6.4 (6.3)	5.3 (5.2)
5	1-0-14:0-De-d18:1, H_2SO_4 salt	122 (dec.)	82	1740 (ν C=O)	$C_{32}H_{64}O_5NS$	68.8 (68.8)	11.7 (11.5)	2.6 (2.5)
6	1-0-14:0-De-d18:1	95	68	1720 (ν C=O)	$C_{32}H_{63}O_3N$	76.0 (75.4)	12.2 (12.5)	2.7 (2.8)
7	1-0-14:0-De-d18:0, H_2SO_4 salt	130-2 (dec.)	78	1740 (ν C=O)	$C_{32}H_{66}O_5NS$	67.1 (68.5)	11.8 (11.9)	2.4 (2.5)
8	1-0-14:0-De-d18:0	96-99	64	1720 (ν C=O)	$C_{32}H_{65}O_3N$	74.9 (75.1)	12.6 (12.8)	2.5 (2.7)

Table 4 contd.

9[h]	3-0-14:0-DL*t*-d18:0, H_2SO_4 salt	166-9 (dec.)	75	1740 (ν C=O)				
10[h]	3-0-14:0-DL*t*-d18:0	89-91	63	1720 (ν C=O)				
11[e,h]	14:0-D*e*-d18:1	94-95	64	1640 (ν C=O) 1545 (δ N-H)				
12[f]	14:0-DL*e*-d18:0	100-1	68	1640 (ν C=O) 1550 (δ N-H)				
13[h]	14:0-DL*t*-d18:0	93-94	58	1610 (ν C=O) 1550 (δ N-H)				
14[g]	24:0-DL*t*-d18:1	93-94	72	1610 (ν C=O) 1545 (δ N-H)	$C_{42}H_{83}O_3N$	76.9 (77.6)	13.3 (12.9)	2.1 (2.2)
15[g]	24:0-DL*t*-d18:0	92-93	65	1610 (ν C=O) 1550 (δ N-H)	$C_{42}H_{85}O_3N$	77.2 (77.3)	12.8 (13.1)	2.0 (2.1)

a) All compounds, with the exception of No. 12, are new;
b) Cl% found, 9.2 (calcd., 9.5);   c) Cl% found, 11.5 (calcd., 12.0);   d) recrystallized many times;
e) reported m.p. for 14:0-DL*e*-d18:1, 86-87[136];   f) reported m.p., 103-4[128];   g) prepared by method of Hammarström[36];
the m.p. of its *e*-isomer was the same as that reported[137];   h) not prepared in sufficient quantity for microanalysis.

CHAPTER 3

STEREOCHEMICAL STUDIES OF SPHINGOID DERIVATIVES

Other authors have separated diastereoisomers of psychosines[138] and sphingosylphosphorylcholines[13] by TLC on plain silica gel, and of DNP derivatives of sphingoids[31] and polyhydroxy fatty acids[139] on silica gel impregnated with borate. Diastereoisomeric N-acetyl sphingoids have been separated by GLC[84]. It is now shown that the modifications described in Chapter 1 (pp. 23 and 36) for these methods may be used for similar chromatographic separations of acyl sphingoids. The results may be used to obtain evidence for the existence of natural diastereoisomeric ceramides. This is also the place to consider possible stereochemical changes in acyl sphingoids and related oxazolines under normal or adverse conditions, and spectroscopic differences between isomers.

1. TLC of isomeric acyl sphingoids and sphingoid-oxazolines

(a) Diastereoisomeric ceramides

Ceramides were synthesized essentially by a known method[36], that of condensing the sphingoid and fatty acid in the presence of a carbodiimide. The byproducts of the synthesis (mainly N-acylureas[140]) were removed by preparative TLC, and the ceramides recrystallized from methanol. Most of the ceramides prepared for TLC (or GLC) analysis, including all of those derived from t-sphingoids or L-2-hydroxy fatty acids, were new compounds so far as is known; some were prepared in quantities large enough for microanalysis (p. 75).

The separation of diastereoisomeric ceramides on TLC plates is illustrated in Figs. 1 to 5, and their R_f values are listed in Table 1. It may be seen that diastereoisomeric NFA ceramides do not separate on a plain TLC plate (Fig. 1). On a plate impregnated with borate (Fig. 2), NFA ceramides derived from either e-d18:1 (lane 2), t-d18:1 (lane 3), or t-d18:0 (lane 5), are retarded. The former effect has been observed before[71]. The latter effect may be ascribed to the complex formed between the borate ion and the two hydroxy groups of the sphingoid. The partial separation observed between diastereoisomers derived from d18:1 (lanes 2 and 3) indicates that the borate ion may complex with the 3-hydroxy group and the double bond. A minor component in lane 6 was left unidentified by others using similar standards[137]. Its persistent similarity to the product of lane 5 under diverse chromatographic conditions suggests that it is also a t-ceramide.

HFA ceramides derived from a DLe- or DLt-sphingoid (d18:1 or d18:0), or a mixture of these, and a racemic 2-hydroxy fatty acid, gave two spots on freshly activated plain TLC plates (Fig. 3, lanes 2 and 3). Similar results have been obtained by others for diastereoisomeric HFA ceramides derived from De-d18:1[36]; the upper and lower spots observed here may be ascribed to HFA ceramides derived from L- and D-hydroxy fatty acids respectively. When representatives of these ceramide classes were prepared from a diastereoisomeric mixture of d18:1 or d18:0, a partial separation on unactivated TLC plates was observed (not illustrated). On plates impregnated with borate, the e- and t-ceramides, with either L- or D-hydroxy fatty acids, were completely separated from each other (Figs. 4 and 5). These results differ from those of NFA ceramides in that e- and t-diastereoisomers derived from d18:1 are separated. This behaviour could be explained by a borate complex formed between the hydroxy group of the fatty acid

Analysis of diastereoisomeric ceramides

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Determination of the most important sphingolipids (particularly sphingomyelins and cerebrosides) is carried out by their hydrolysis to free ceramides (N-acyl sphingoids[1]) which are then subjected to chromatography, usually after derivatisation. Such a procedure is inadequate when interest centres on the presence of free ceramides that are of diagnostic significance in food chemistry[2] or clinical biochemistry[3] as minor constituents of some natural fats and oils. A simple method of t.l.c. suitable for the analysis of sphingomyelins and cerebrosides[4] does not separate free ceramides from the latter or requires additional operations[3] that make routine assays impracticable and fail to distinguish between different classes of ceramides.

A convenient method of t.l.c. employs silica gel G (Merck) and a mixture of chloroform/carbon tetrachloride/methanol/ water, 50:50:12:0·5 v/v (eluting agent A). Ceramides in the region of R_f 0·28-0·56 are well separated from the much slower

cerebrosides and faster cholesterol bands, and sub-fractionation of structurally or stereochemically different classes of ceramides is satisfactory for the purposes of quantitative analysis of preparative t.l.c.

Three types of interfering lipid have been found. Free fatty acids can be eliminated either by a 1 cm thick bar⁻ⁱ⁻ produced at the bottom of the plate by spraying it with a 10 per cent solution of sodium carbonate or more satisfactorily by replacing water in the eluting agent by 15N ammonia.` Neither of these procedures affects the R_f of neutral lipids. Monoglycerides have the same R_f values as the relatively rare ceramides derived from α-hydroxy-acids, and can be recognised by colour tests for esters[6] or vicinal diols,[7] estimation of ester groups[8] or infrared spectroscopy. In some commercial ceramides prepared by oxidative degradation of cerebrosides followed by hydrolysis,[9] one encounters 'polyols' identified as $CH_3(CH_2)_{12}CH$

Table 2 **Retention times of diastereoisomeric ceramides,[a] relative to N-stearoyl *erythro*-sphinganine**

N-acyl[b]	Ceramides derived from sphingenine						Ceramides derived from sphinganine					
	unsubstituted acid		hydroxy acids				unsubstituted acid		hydroxy acids			
	e	t	L'e	L't	D'e	D't	e	t	L'e	L't	D'e	D't
14:0	0·47	0·43			0·47		0·50	0·45				
16:0	0·66	0·60	0·68	0·66	0·68	0·56	0·70	0·63	0·68	0·63	0·68	0·56
18:0	0·93	0·85	0·96		0·96	0·79	1·00	0·89	0·96		0·96	0·79
20:0	1·35	1·24	1·35		1·35	1·14	1·45	1·29	1·37		1·37	1·14
22:0	1·99	1·82	2·00		2·00	1·68	2·14	1·90	2·03		2·03	1·67
24:0	2·90	2·66	2·86		2·86	2·43	3·12	2·78	2·90		2·90	2·42
24:1							3·07	2·74				
26:0	4·22				4·14				4·20		4·20	3·51

[a] L or D and e or t indicate the configuration of the 2-hydroxy fatty acid and sphingoid, respectively;
[b] number of C atoms and unsaturation are indicated

$=CH\text{-}CHOH\text{-}CH(NHCOR)\text{-}CH_2O\text{-}CH(CH_2OH)\text{-}OCH(CH_2OH)_2$ by infrared and mass spectroscopy and conversion into ceramides on prolonged hydrolysis. In many cases (e.g. study of synthetic ceramides) monoglycerides and polyols may be ignored; otherwise they may be separated from ceramides by two-dimensional chromatography first with eluting agent A, then ether/acetone/15N ammonia, 80:20:2 v/v.

Stereochemical separation of various sphingoid derivatives by t.l.c. on silica gel[10,11] or silica gel impregnated with borate[12,13] has been extended to ceramides available as commercial samples or prepared from brain[13,14] or milk[15,16] or by synthesis.[17] Free *threo* ceramides move slower than *erythro* diastereoisomers. Although separation of diastereoisomeric ceramides derived from unsubstituted fatty acids and unsaturated sphingoids is incomplete (presumably owing to the group), 2-hydroxy-acid derivatives of both saturated and unsaturated sphingoids are easily separated. It is known that *erythro* ceramides derived from L-acids move faster than those derived from D-acids,[12] now it is found that the same rule applies to *threo* ceramides and that D-*erythro* moves faster than L-*threo*. R_f values on plain and borate-treated t.l.c. plates developed with chloroform containing 8 per cent v/v methanol (eluting agent B) shown in Table 1 are given relative to the R_f (0·50) of racemic N-stearoyl-*erythro*-sphinganine.

Separation of diastereoisomeric sphingoids as N-acetyl derivatives by g.l.c.[18] can be extended to ceramides. Retention times relative to N-stearoyl *erythro*-sphinganine in the range on a column (3·3mm by 3m, glass) containing 1 per cent GE SE-30 (GC grade) plus 1 per cent OV-17 on 100-120mesh Gas Chrom Q are shown in Table 2. It is seen that *threo* compounds emerge before *erythro* isomers.

Table 1 **R_f values of ceramides relatively to N-stearoyl *erythro*-sphinganine on plain t.l.c. plates or plates impregnated with 10 per cent w/w borate, developed with 8 per cent CH₃OH in CHCl₃.**

Ceramide moieties[a]		Relative R_f values on	
N-acyl	racemic sphingoid	plain plates	borate plates
24:0	e-18:1	1·12	0·84
24:0	t-18:1	1·10	0·78
24:0	e-18:0	1·12	1·12
24:0	t-18:0	1·10	0·86
14:0	e-18:1	0·92	0·72
14:0	t-18:1	0·90	0·68
14:0	e-18:0	0·92	0·92
14:0	t-18:0	0·90	0·74
L-h 26:0	e-18:1	1·02	0·72
D-h 26:0	e-18:1	0·76	0·60
L-h 26:0	t-18:1	1·00[b]	0·58
D-h 26:0	t-18:1	0·70	0·36
L-h 26:0	e-18:0	1·00	1·00
D-h 26:0	e-18:0	0·74	0·74
L-h 26:0	t-18:0	0·98	0·58
D-h 26:0	t-18:0	0·70	0·36
L-h 16:0	e-18:1	0·90	0·64
D-h 16:0	e-18:1	0·64	0·54
L-h 16:0	t-18:1	0·84	0·50
D-h 16:0	t-18:1	0·56	0·28
L-h 16:0	e-18:0	0·90	0·88
D-h 16:0	e-18:0	0·64	0·66
L-h 16:0	t-18:0	0·84	0·50
D-h 16:0	t-18:0	0·56	0·28

[a] e = *erythro* t = *threo* h = α-hydroxy;
[b] absolute value of R_f = 0·50

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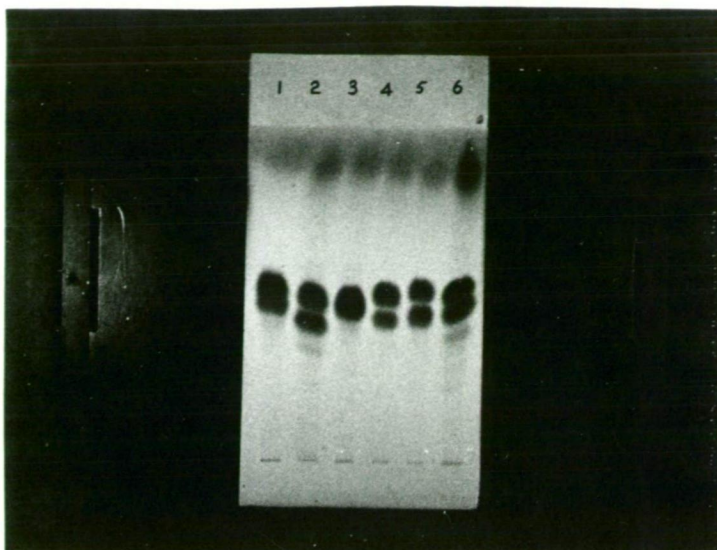
Fig. 1.   TLC plate illustrating the separation of isomeric
ceramides.   Developing solvent, chloroform-carbon tetrachloride-
methanol-water (50:50:12:0.5).   1, natural ceramides from brain
sphingomyelin; 2, 24:0-DLe-d18:1 (upper spot) and 14:0-De-d18:1
(lower spot); 3, 24:0-DLt-d18:1; 4, 24:0-DLe-d18:0 (upper spot) and
14:0-DLe-d18:0 (lower spot); 5, 24:0-DLt-d18:0 (upper spot) and
14:0-DLt-d18:0 (lower spot); 6, standard ceramides, 24:0-DLe-d18:0
(upper spot) and 16:0-DLe-d18:0 (lower spot).
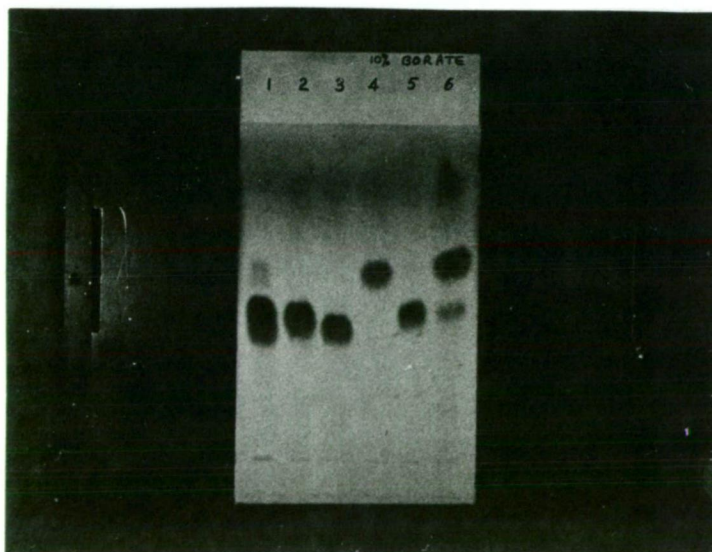
Fig. 2.   TLC plate illustrating the separation of diastereoisomeric
NFA ceramides; the silica gel is impregnated with borate (10% W/w).
Developing solvent, chloroform-carbon tetrachloride-methanol-water
(50:50:12:0.5).   1, natural ceramides from brain sphingomyelin;
2, 24:0-DLe-d18:1; 3, 24:0-DLt-d18:1; 4, 24:0-DLe-d18:0;
5, 24:0-DLt-d18:0; 6, standard ceramide, 24:0-DLe-d18:0.
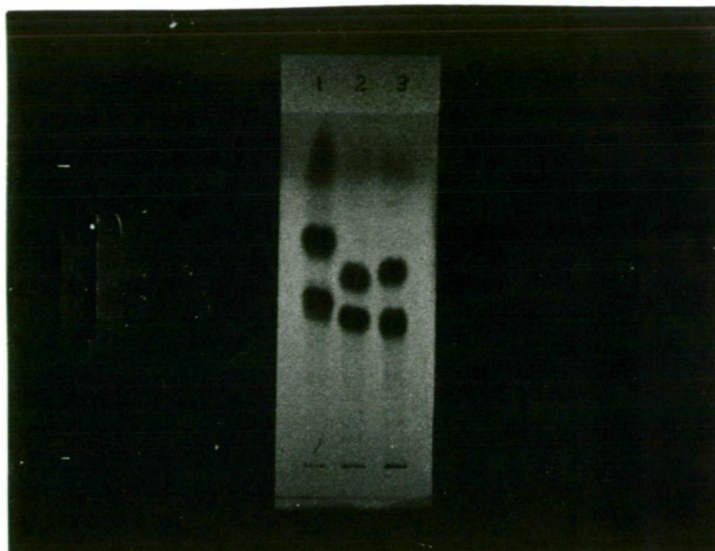
Fig. 3.   TLC plate illustrating the separation of diastereoisomeric
HFA ceramides.   Developing solvent, chloroform-methanol (92:8).
1, natural mixture of NFA ceramides (upper spot) and HFA ceramides
(lower spot);   2, L-h18:0-DL(e+t)-d18:1 (upper spot) and D-h18:0-
DL(e+t)-d18:1 (lower spot);   3, L-h18:0-DL(e+t)-d18:0 (upper spot) and
D-h18:0-DL(e+t)-d18:0 (lower spot).
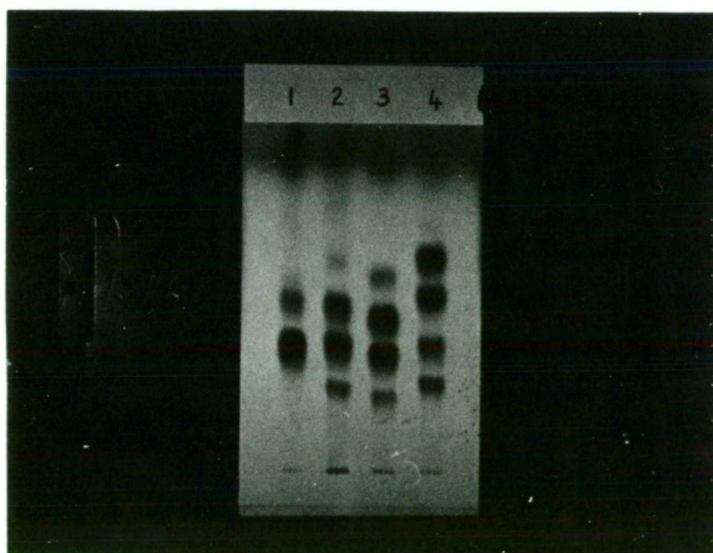
Fig. 4.   TLC plate illustrating mixtures of diastereoisomeric
HFA ceramides;   the silica gel is impregnated with borate (10%, ^{W}/w).
Developing solvent, chloroform-methanol (85:15).   1, natural mixture
of HFA ceramides;   2, DL-h24:0-DL(e+t)-d18:1;   3, DL-h18:0-DL(e+t)-
d18:1;   4, DL-h26:0-DL(e+t)-d18:0.
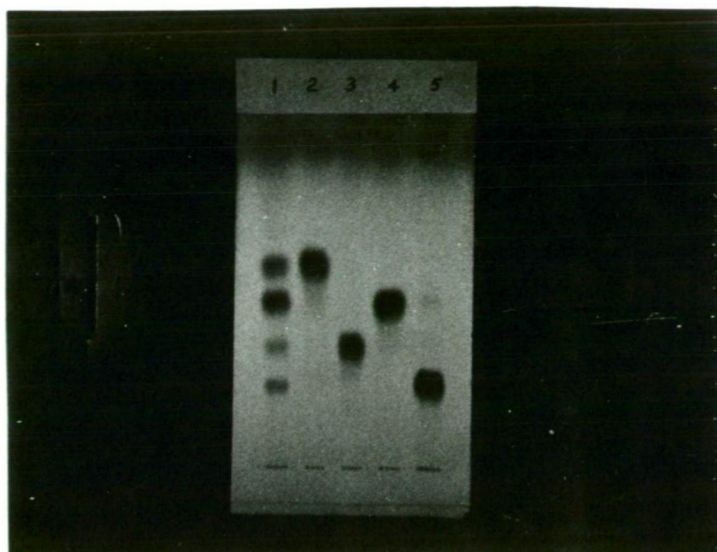
Fig. 5.   TLC plate illustrating the ceramides which have been
isolated from a diastereoisomeric mixture and recrystallized; the
silica gel is impregnated with borate (10%, W/w).   Developing
solvent, chloroform-methanol (85:15).   1, DL-h16:0-DL(e+t)-d18:0;
2, L-h16:0-DLe-d18:0; 3, L-h16:0-DLt-d18:0; 4, D-h16:0-DLe-d18:0;
5, D-h16:0-DLt-d18:0.
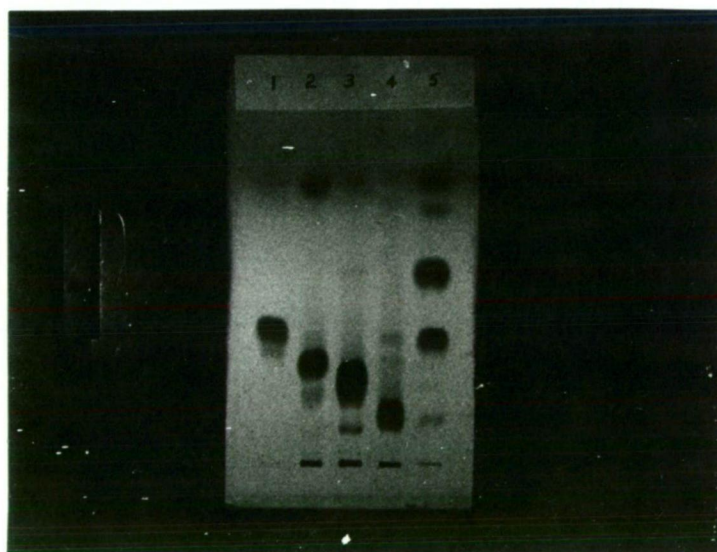
Fig. 6.   TLC plate illustrating the relative chromatographic
mobilities of isomeric acyl sphingoids; the silica gel is impregnated
with borate (10%, W/w).   Developing solvent, chloroform-methanol (92:8).
1, 14:0-De-d18:1 (the faint spot below is probably a trace of the
t-isomer); 2, 1-0-14:0-De-d18:1 (upper spot) and 3-0-14:0-De-d18:1
(lower spot); 3, 1-0-14:0-DLe-d18:0 (upper spot) and 3-0-14:0-DLe-d18:0
(lower spot); 4, 3-0-14:0-DLt-d18:0; 5, 14:0-DLe-d18:0 (upper spot)
and 14:0-DLt-d18:0 (lower spot).

Table 1

R_f values of ceramides and O-acyl sphingoids, relative to 18:0-DL$\underline{e}$-d18:0, on plain TLC plates (column A), or plates impregnated with 10% ^{W}/w borate (column B).   For plates developed with 8% CH_3OH in $CHCl_3$, 18:0-DL$\underline{e}$-d18:0 moves to R_f 0.50.

Compound name	R_f values	
	A	B
24:0-DL$\underline{e}$-d18:1	1.12	0.84
24:0-DL$\underline{t}$-d18:1	1.10	0.78
24:0-DL$\underline{e}$-d18:0	1.12	1.12
24:0-DL$\underline{t}$-d18:0	1.10	0.86
14:0-DL$\underline{e}$-d18:1	0.92	0.72
14:0-DL$\underline{t}$-d18:1	0.90	0.68
14:0-DL$\underline{e}$-d18:0	0.92	0.92
14:0-DL$\underline{t}$-d18:0	0.90	0.74
1-0-14:0-DL$\underline{e}$-d18:1	0.74	0.54
3-0-14:0-DL$\underline{e}$-d18:1	0.58	0.36
1-0-14:0-DL$\underline{e}$-d18:0	0.64	0.44
3-0-14:0-DL$\underline{e}$-d18:0	0.48	0.28
3-0-14:0-DL$\underline{t}$-d18:0	0.48	0.28
L-h 26:0-DL$\underline{e}$-d18:1	1.02	0.72
D-h 26:0-DL$\underline{e}$-d18:1	0.76	0.60
L-h 26:0-DL$\underline{t}$-d18:1	1.00	0.58
D-h 26:0-DL$\underline{t}$-d18:1	0.70	0.36
L-h 26:0-DL$\underline{e}$-d18:0	1.00	1.00
D-h 26:0-DL$\underline{e}$-d18:0	0.74	0.74
L-h 26:0-DL$\underline{t}$-d18:0	0.98	0.58
D-h 26:0-DL$\underline{t}$-d18:0	0.70	0.36
L-h 16:0-DL$\underline{e}$-d18:1	0.90	0.64
D-h 16:0-DL$\underline{e}$-d18:1	0.64	0.54
L-h 16:0-DL$\underline{t}$-d18:1	0.84	0.50
D-h 16:0-DL$\underline{t}$-d18:1	0.56	0.28
L-h 16:0-DL$\underline{e}$-d18:0	0.90	0.88
D-h 16:0-DL$\underline{e}$-d18:0	0.64	0.66
L-h 16:0-DL$\underline{t}$-d18:0	0.84	0.50
D-h 16:0-DL$\underline{t}$-d18:0	0.56	0.28

and the amide nitrogen[141]. Scale models based on the known structure of 24:0-t18:0[142] showed that the hydroxy group of the fatty acid and either of the hydroxy groups of the sphingoid were too distant for borate complex formation.

These results show that the TLC detection of t-NFA ceramides in a diastereoisomeric mixture is difficult. On the other hand, the detection of t-HFA ceramides derived from 2-D-hydroxy fatty acids, and e- or t-HFA ceramides derived from 2-L-hydroxy fatty acids, is possible. In natural HFA ceramides neither t-sphingoids nor L-hydroxy fatty acids were detected by TLC (Figs. 3 and 4, lane 1 in each).

The separation of ceramides derived from a DLe- or DLt-sphingoid, and a normal, 2-L-hydroxy or 2-D-hydroxy fatty acid, was attempted with TLC plates impregnated with borate and cellulose. The results were negative, but the mixtures may be resolved into their optical isomers by a recently published method[143] which involves derivatisation with L(+) acetylmandeloyl chloride followed by TLC separation.

(b) Isomeric O-acyl sphingoids and related oxazolines

The TLC separation of O-acyl sphingoids from the parent ceramides on silica gel impregnated with borate is illustrated by Fig. 6. This kind of plate was found to give more effective separations than plain plates. Both 1-O-acyl sphingoids (major components, lanes 2 and 3) and 3-O-acyl sphingoids (major component, lane 4) are retarded by borate; the latter move the slowest since they contain a primary hydroxyl group. The 3-O-acyl derivatives of e-d18:1 and e-d18:0 are represented by the slower moving minor spots of lane 2 and lane 3 respectively. The R_f values of O-acyl sphingoids are compared to those of ceramides in Table 1.

Sphingoid-oxazolines are comparatively more non-polar than

acyl sphingoids; on TLC plates used to analyse ceramides, sphingoid-oxazolines move close to the solvent front. A solvent system of 2% methanol in chloroform is more suitable for their analysis by TLC, and as illustrated (Fig. 14), isomers derived from e-d18:1, e-d18:0, and t-d18:0 are separated. Borate has no complexing effect on these compounds, which is understandable since one of the hydroxy groups and the amide nitrogen of the parent ceramides are utilised in ring formation.

2. GLC of diastereoisomeric ceramides

Mixtures of ceramides, synthesised from diastereoisomeric sphingoids and normal or racemic 2-hydroxy acids , were analysed essentially by the GLC method described in Chapter 1 (p.), with the exception that the helium flow rate was faster, 90 ml/minute. In general, t-ceramides emerged before e-ceramides. In the case of NFA ceramides derived from d18:1, the separation of diastereoisomers was incomplete, and a t-isomer was recognized as a minor component by a bump on the peak for the e-isomer (Fig. 7). Diastereoisomeric NFA ceramides derived from d18:0 were completely separated (not illustrated). In the case of HFA ceramides derived from d18:1, a t-isomer containing a D-2-hydroxy acid was completely separated from the other isomers (Fig. 8). The t-isomer containing an L-2-hydroxy acid was part of the peak for the e-isomers, though when chromatographed separately its retention time was slightly lower. Diastereoisomeric HFA ceramides derived from d18:0 gave similar results (not illustrated); however, the separation of these was slightly improved on a column of 3% SE-30 on 100-120 mesh Gas Chrom Q, and the t-isomer containing an L-2-hydroxy acid was also distinguished (Fig. 9).

The retention times for some of the synthetic ceramides
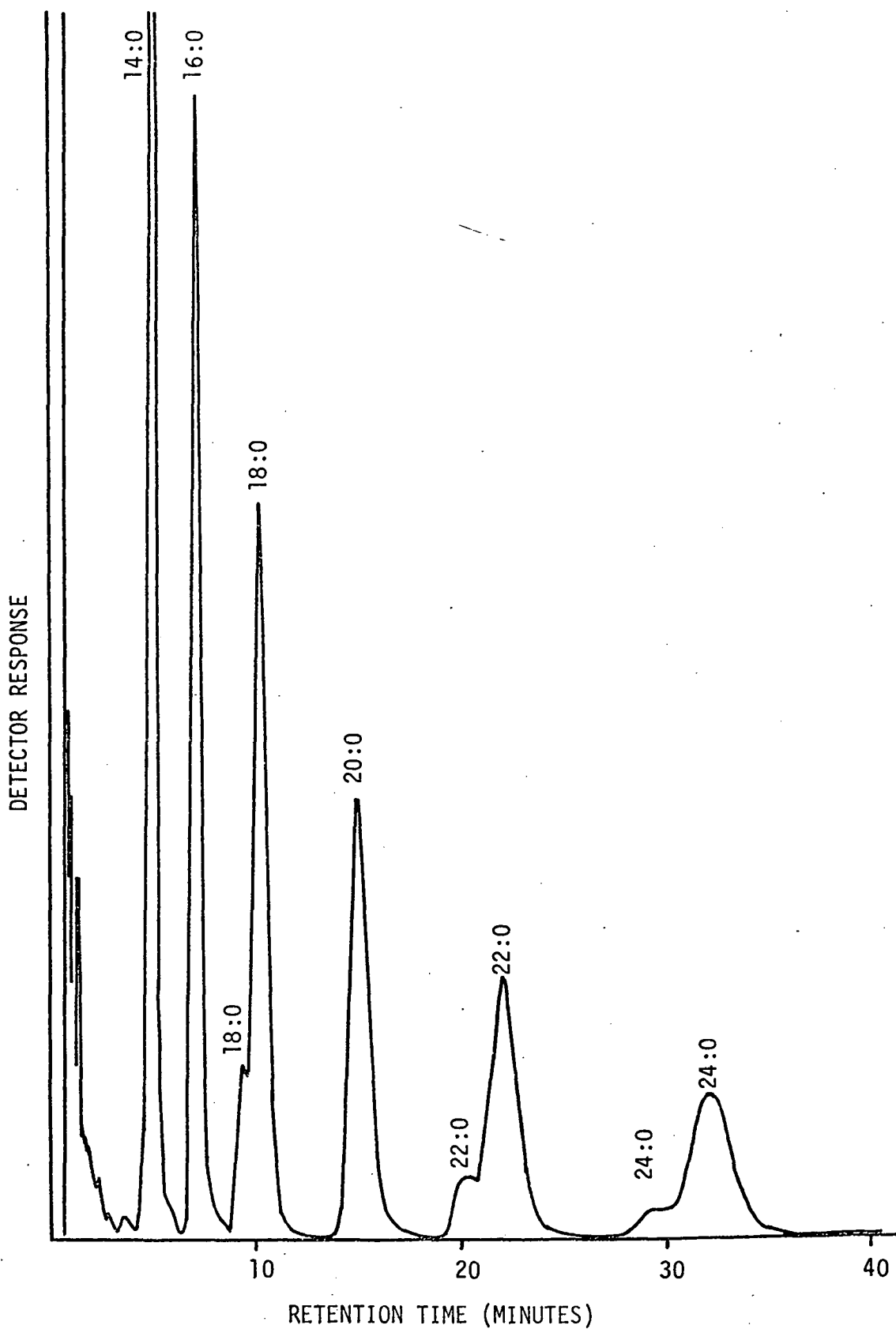
Fig. 7. GLC separation of synthetic NFA ceramides. The major peaks represent derivatives of e-d18:1, and the minor peaks represent derivatives of t-d18:1 (the fatty acid of each is indicated). A column (3m x 3.3mm) of 1% GE SE-30 plus 1% OV-17 on 100-120 mesh Gas Chrom Q, temperature 300°, and He flow rate 90 ml/min, was used.
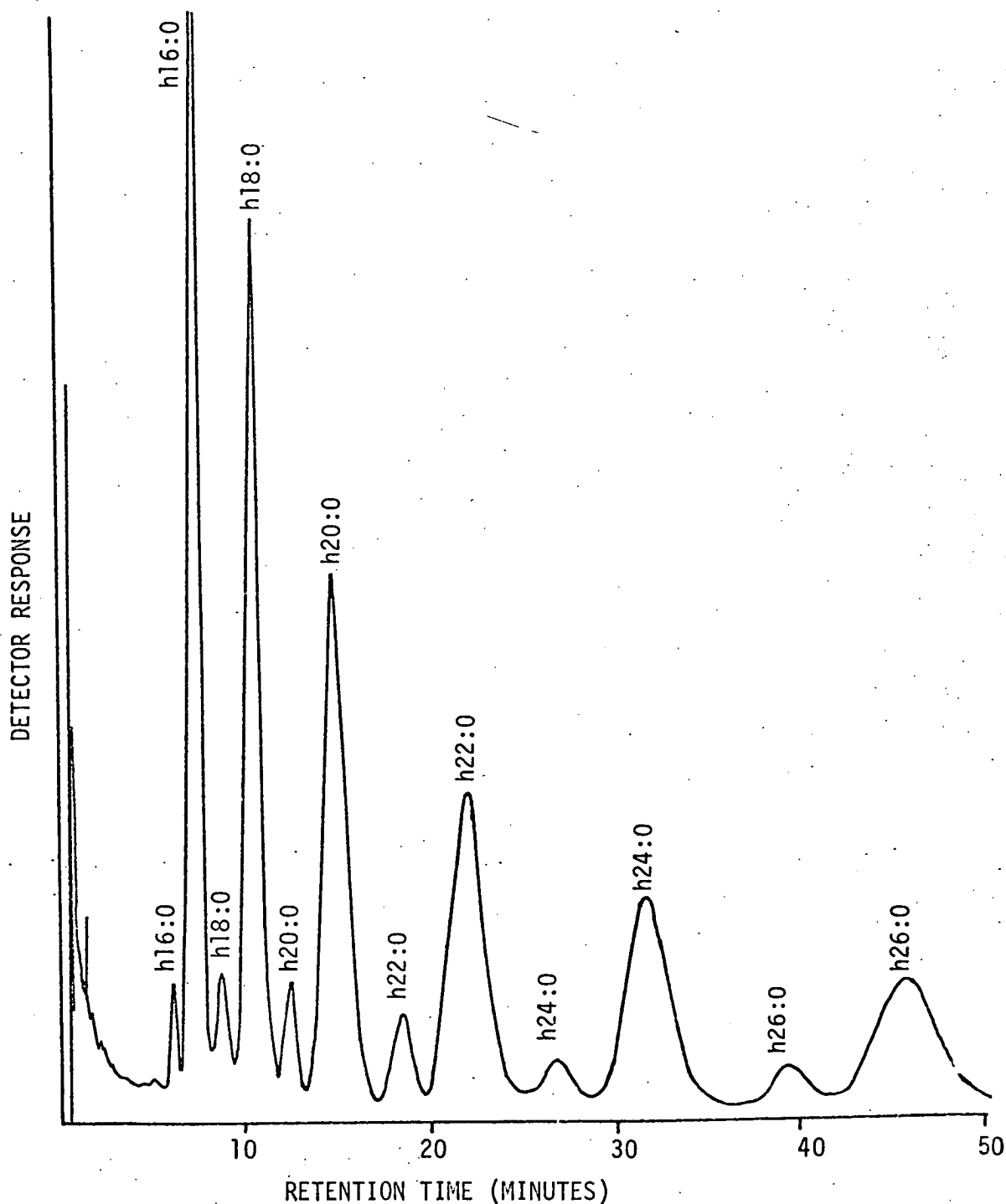
Fig. 8. GLC separation of synthetic HFA ceramides. The minor peaks represent compounds derived from t-d18:1 and 2-D-hydroxy fatty acids, and the major peaks represent the isomers derived from e-d18:1 and 2-DL-hydroxy fatty acids, plus t-d18:1 and 2-L-hydroxy fatty acids (the fatty acid of each is illustrated). The GLC conditions are given in Fig. 7.
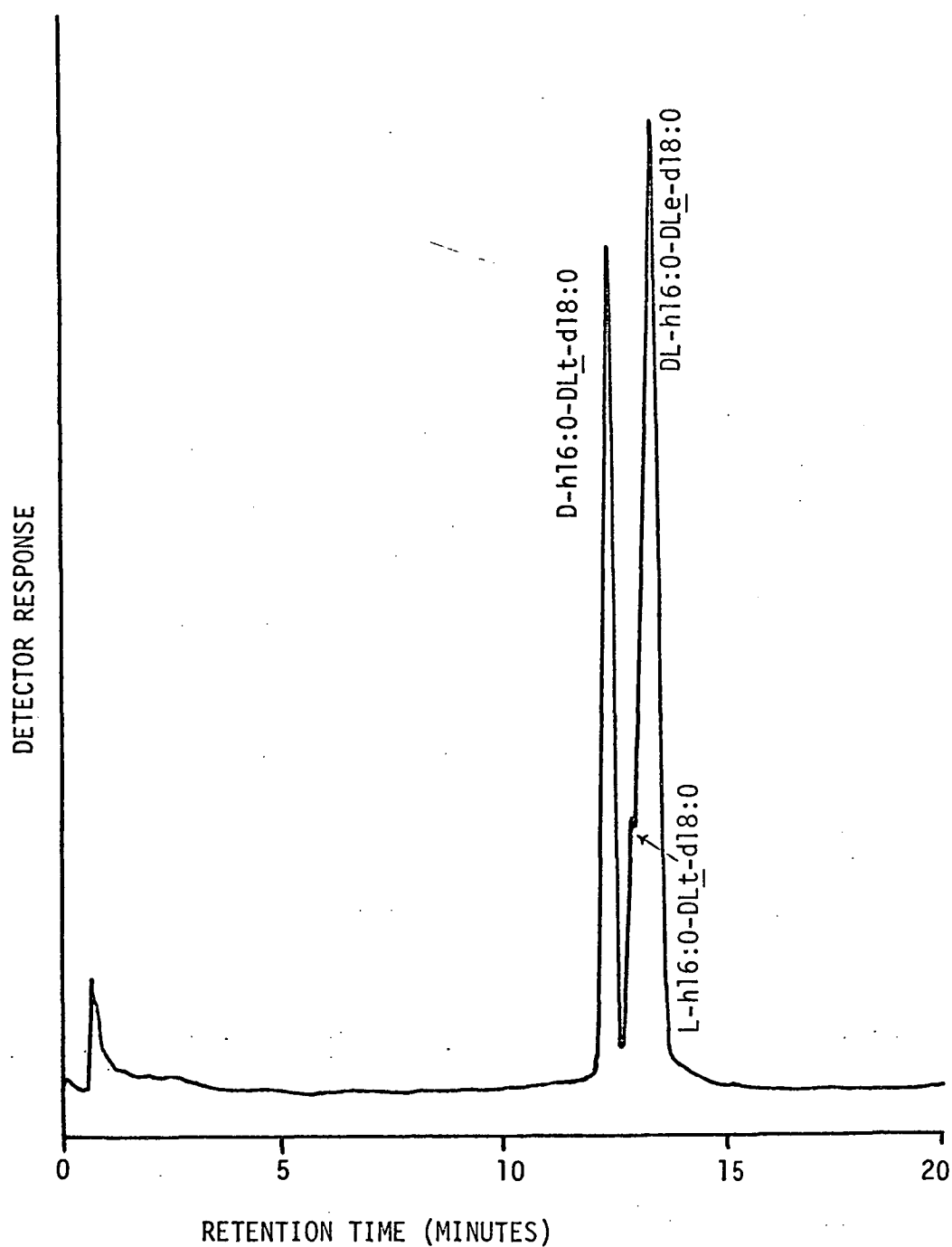
Fig. 9. GLC separation of the isomers of DL-h16:0-rac-d18:0. A
column (2m x 3mm) of 3% GE SE-30 on 100-120 mesh Gas Chrom Q,
temperature 285°, and N2 flow rate 40 ml/min., was used.
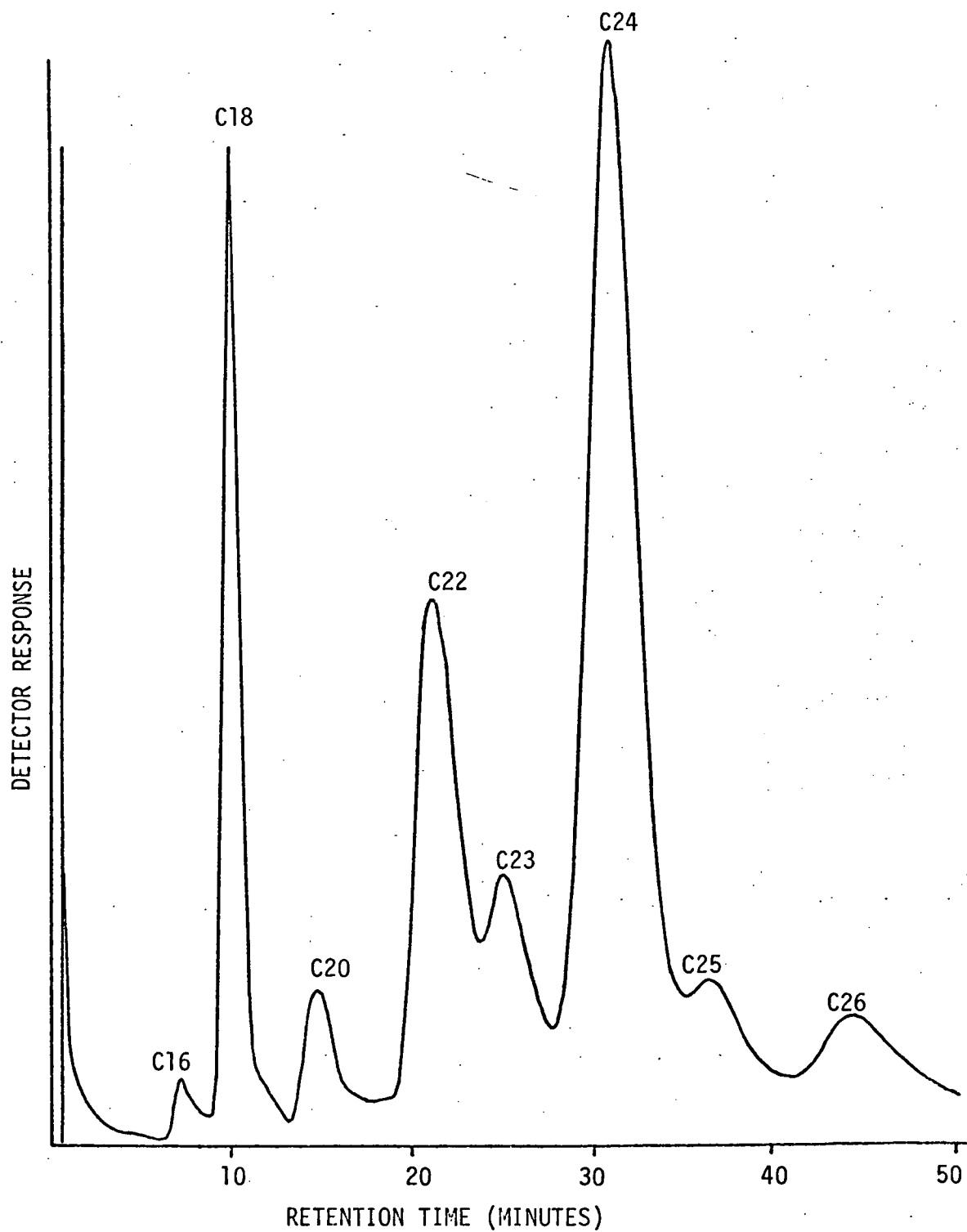
Fig. 10.   GLC separation of ceramides derived from brain NFA cerebrosides. The carbon chain lengths of the fatty acids are illustrated.   GLC conditions are given in Fig. 7.
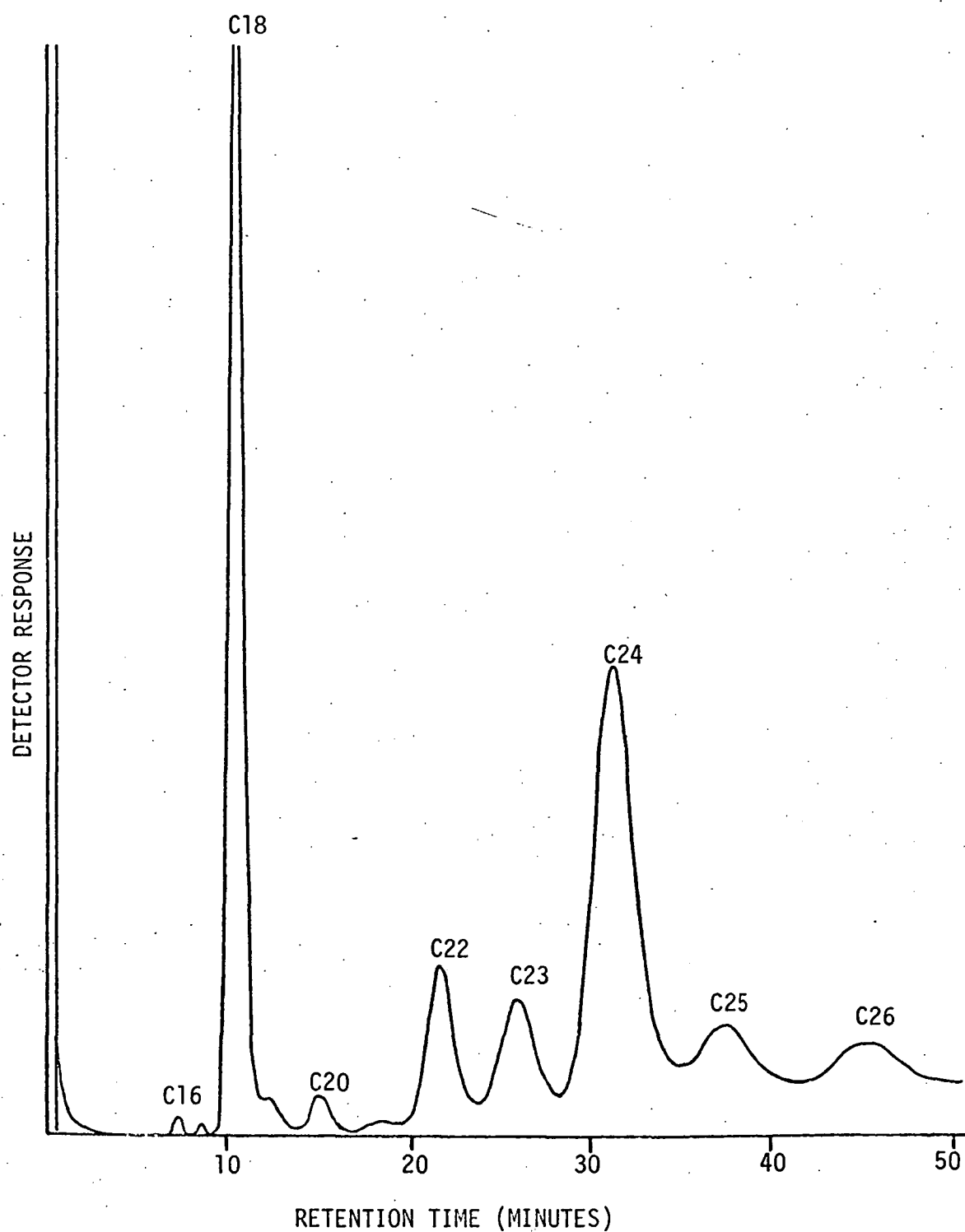
Fig. 11. GLC separation of ceramides derived from brain HFA cerebrosides. The carbon chain lengths of the fatty acids are illustrated. GLC conditions are given in Fig. 7.

Table 2

Retention times of diastereoisomeric ceramides, relative to 18:0-DLe-d18:0

Fatty acid	Ceramides derived from DL-d18:1						Ceramides derived from DL-d18:0					
	NFA derivatives		HFA derivatives[*]				NFA derivatives		HFA derivatives[*]			
	e	t	L'e	L't	D'e	D't	e	t	L'e	L't	D'e	D't
14:0	0.47	0.43			0.47		0.50	0.45				
16:0	0.66	0.60	0.68	0.66	0.68	0.56	0.70	0.63	0.68	0.63	0.68	0.56
18:0	0.93	0.85	0.96		0.96	0.79	1.00	0.89	0.96		0.96	0.79
20:0	1.35	1.24	1.35		1.35	1.14	1.45	1.29	1.37		1.37	1.14
22:0	1.99	1.82	2.00		2.00	1.68	2.14	1.90	2.03		2.03	1.67
24:0	2.90	2.66	2.86		2.86	2.43	3.12	2.78	2.90		2.90	2.42
24:1							3.07	2.74				
26:0	4.22		4.14		4.14	3.55			4.20		4.20	3.51

[*]L' or D' and e or t indicates the configuration of the 2-hydroxy fatty acid and sphingoid, respectively.

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investigated, relative to 18:0-DL_e-d18:0, are given in Table 2. The similarity of these values for either e-ceramides or t-ceramides containing the same number of carbon atoms is evident. Analysis of mixtures of ceramides of natural origin would require group separation followed by GLC-mass spectrometry.

The methods for producing ceramides from other sphingolipids have not been investigated with regard to isomerisation. Thus natural brain ceramides, ceramides derived from brain sphingomyelin either enzymatically[58] or by acetic acid hydrolysis[57], and ceramides derived from brain cerebrosides by periodate oxidation followed by reduction and mild acid hydrolysis[61], were analysed by this GLC method. The results for NFA ceramides and HFA ceramides derived from cerebrosides are illustrated (Figs. 10 and 11 respectively); t-ceramides were not detected. Alkaline hydrolysis of each ceramide fraction produced e-sphingoids only as determined by GLC (Ch. 1, p.36).

3. Stereochemical changes during hydrolysis of sphingoid derivatives

Acyl sphingoids and related oxazolines derived from e-d18:1, t-d18:1, e-d18:0, or t-d18:0 were subjected to hydrolysis with acid[98] or strong alkali[61], and the sphingoids analysed as DNP derivatives on TLC plates impregnated with borate[31]. Acid hydrolysis of N-acyl-, 3-0-acyl-, and 2,3-substituted oxazoline derivatives of e-d18:1 liberated the sphingoid unchanged, its diastereoisomer t-d18:1, dehydration products[144], and 0-methyl derivatives[145,146]. All other sphingoid derivatives underwent acid hydrolysis with little degradation or stereochemical changes to the sphingoids. Alkaline hydrolysis liberated only e-sphingoids in all cases, with the exception of the sphingoid-oxazolines which were not affected by this treatment.

4. Isomerisation of sphingoid-oxazolines in solution

Pure sphingoid-oxazolines were stable when stored dry at 0°, but when dissolved in dry solvents such as alcohol, chloroform, or ether, the compounds began to isomerise. The presence of alkali and/or water did not change the rate of isomerisation appreciably. After several days of standing at room temperature, when no further change could be detected, mixtures of isomeric oxazolines in equilibrium were obtained. As illustrated (Figs. 12 and 13), the configurations of the sphingoids (determined by TLC of the DNP derivatives[31]) did not change during isomerisation. The isomers most favoured were I (Fig. 12) and III (Fig. 13), derived from e- and t-sphingoids respectively; these were the principal compounds obtained from the condensation of the sphingoids with imidic acid ester hydrochlorides (Ch. 2, p.70). The trans-oxazolines (III) seemed to be more stable than the cis-oxazolines (I), judging by the relative intensities of the TLC spots for each isomeric mixture (Fig. 14). The ratios I/II and III/IV, determined by weight after TLC separation, were approximately 70/30 and 95/5 respectively. During the isomerisation, and for several weeks after the reaction was complete, amides and esters of the parent compounds were not produced, as judged by TLC. The isomers could be separated by TLC (with some degradation to acyl sphingoids) and recrystallized without isomerisation. The processes of Figs. 12 and 13 resumed only when the separated isomers were redissolved.

The oxazoline (II, $R=CH_3(CH_2)_{14}$), gave indefinite compounds when it was used by other workers[25] for the synthesis of dihydrosphingomyelin; the instability observed here for this type of oxazoline may account for this. The synthesis was eventually carried out successfully with the 2-phenyl analogue.
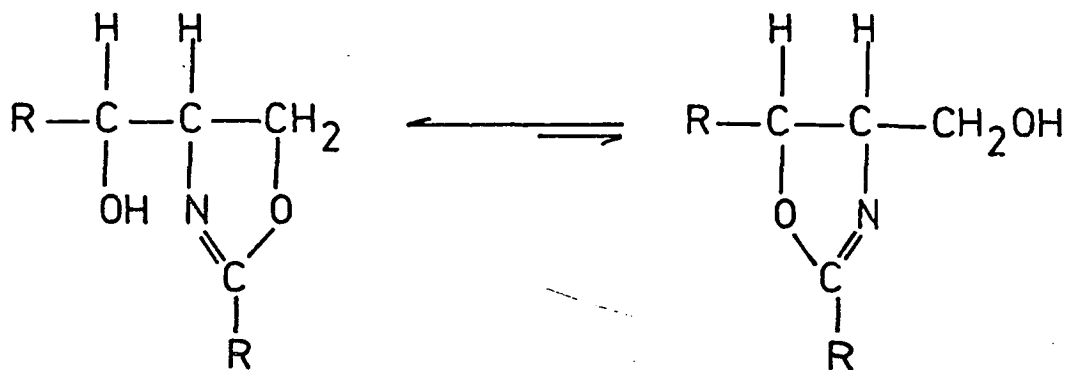
Fig. 12
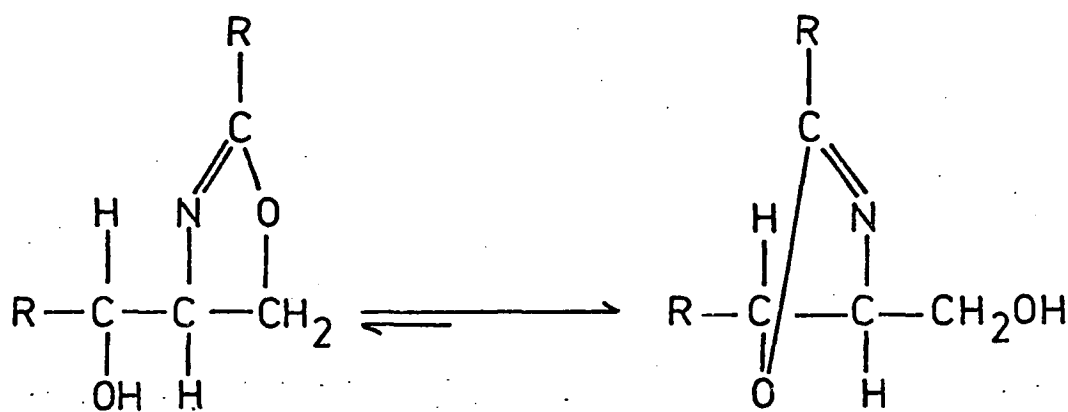
Fig. 13

Figs. 12,13.   Sphingoid-oxazolines in equilibrium in solution (derivatives of D-sphingoids only are illustrated for convenience). R = long-chain alkyl group.
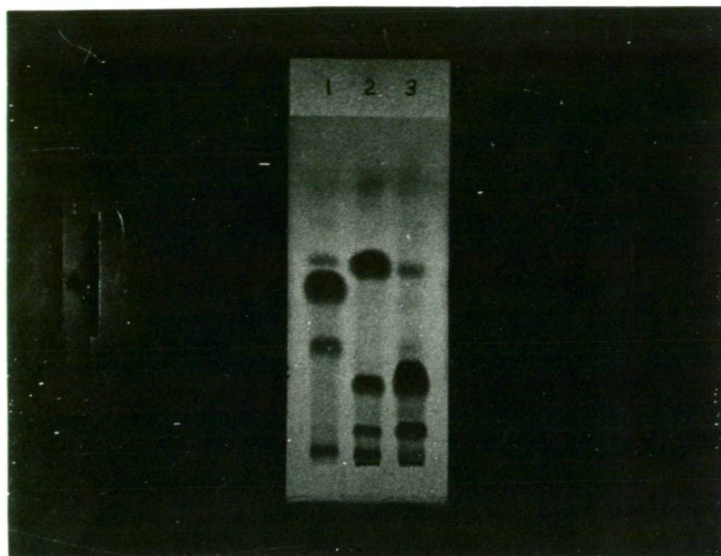
Fig. 14.   TLC plate illustrating isomerised sphingoid-
oxazolines after standing in solution for several days; the intense
spots are the more stable isomers.   Developing solvent, chloroform-
methanol (98:2).   1, oxazolines derived from e-d18:1 and 14:0
(R_f 0.50, 0.33); 2, oxazolines derived from e-d18:0 and 14:0
(R_f 0.56, 0.23); 3, oxazolines derived from t-d18:0 and 14:0
(R_f 0.23, 0.56).

5.   Spectroscopic analysis of sphingoid derivatives

The detection of acyl sphingoids and sphingoid-oxazolines in ceramides, and the differentiation of isomeric sphingoid derivatives, was possible by the use of IR spectroscopy.   Mass spectroscopy was of little use, due to the similarity in spectra.   Apart from a few IR spectra for e-ceramides (in KBr discs) published by other workers[36,137] no data are available on diastereoisomers and related sphingoid derivatives.   These are recorded in this section.

(a) Diastereoisomeric acyl sphingoids

The IR spectra (in Nujol) for 24:0-DLe-d18:0 and 24:0-DLt-d18:0 (Figs. 15 and 16 respectively) are typical of those for other diastereoisomeric NFA ceramides.   The main differences between the two are the positions of the C=O stretch, the combined N-H and O-H stretch, and the C-O stretch.   These differences may be attributed to the degree of intramolecular hydrogen bonding between the 3-hydroxy group of the sphingoid and the amide group.   The main absorption bands for e-NFA ceramides (also for the natural derivatives) occur at 3260 cm^{-1} (ν N-H and ν O-H), 3080 cm^{-1} (amide II overtone of highly associated amides[147]), 1640 cm^{-1} (amide I band, primarily ν C=O), 1550 cm^{-1} (amide II band, a mixture of 60% δ N-H and 40% ν C-N[148]), 1280 cm^{-1} (amide III band, a mixture of 40% ν C-N, 30% δ N-H, and 20% ν C-C[148]), and 1190 cm^{-1} to 1020 cm^{-1} (ν C-O).   For t-NFA ceramides, the respective frequencies are 3440 cm^{-1} plus 3350 cm^{-1}, 3080 cm^{-1} (much decreased in intensity), 1610 cm^{-1}, 1550 cm^{-1}, 1310 cm^{-1}, and 1110 cm^{-1} plus 1080 cm^{-1}.   Diastereoisomers derived from d18:1 also absorbed at 960 cm^{-1} (δ C-H for the trans-double bond).   All these data were similar in both Nujol and KBr.   In some spectra, including those of

both natural and synthetic ceramides, there was observed splitting of
the amide I and amide II bands. The spectrum for 14:0-De-d18:1
(Fig. 17), which was run under the same conditions as those reproduced
in Figs. 15 and 16, shows absorption at 1643 cm^{-1}, 1615 cm^{-1}, 1566 cm^{-1},
and 1544 cm^{-1}.

Diastereoisomeric HFA ceramides could be distinguished by noting
the positions of the amide I and amide II bands, the intensity of the
amide II overtone, and the shape of the combined N-H and O-H stretch.
Apart from these differences the spectra were much the same. The
amide I and amide II bands for the four isomers of (L+D)-h16:0-(e+t)-
d18:0 are respectively 1645 cm^{-1} and 1545 cm^{-1} (L-e), 1645 cm^{-1} and
1530 cm^{-1} (L-t), 1620 cm^{-1} and 1545 cm^{-1} (D-e), and 1615 cm^{-1} and
1545 cm^{-1} (D-t). The amide II overtone at 3070 cm^{-1} was present only
in the spectra for the e-derivatives.

The spectra for O-acyl sphingoids are characterised by sharp peaks
for ν O-H, ν N-H, ν C=O, ν C-O-C, and ν C-O. For example, 1-O-14:0-
e-d18:1 absorbed at 3320 cm^{-1} plus 3250 cm^{-1} (ν O-H, ν N-H), 1720 cm^{-1}
(ν C=O), 1600 cm^{-1} (δ N-H), 1172 cm^{-1} (ν C-O-C), 1032 cm^{-1} (ν C-O,
lowered from the usual position of 1100 cm^{-1} by α-unsaturation and
α-branching), and 963 cm^{-1} (δ C-H for the trans-double bond). The
presence of these compounds in ceramides are easily determined by the
ester carbonyl stretch.

(b) Sphingoid-oxazolines

The IR spectra of isomeric sphingoid-oxazolines (in Nujol) are
distinguished by the positions of their C=N and O-H stretching modes,
and the region between 1300 cm^{-1} and 1000 cm^{-1}. With reference to
Figs. 12 and 13 (p.92), of the four possible isomers only
representatives of the oxazoline (I) gave a value of 1667 cm^{-1} for

ν C=N (e.g. Figs. 18 and 19). The other isomers (II, III, and IV) gave a lower value, 1656 cm^{-1} for ν C=N (e.g. Fig. 20 for a representative of III), due to the decreased association with the hydroxyl hydrogen. Other workers[25,149] have reported ν C=N values of 1665 cm^{-1} and 1667 cm^{-1} for representatives of (II); in the former case[25], isomerisation to (I) may have occurred, as was mentioned before (p.91).

The position of ν O-H varies only slightly for each isomer, occurring between 3250 cm^{-1} and 3200 cm^{-1} for the oxazolines derived from d18:0, and 3150 cm^{-1} for the oxazoline (I) derived from d18:1. Other workers[25,149] have reported ν O-H values of 3460 cm^{-1} and 3333 cm^{-1} for representatives of (II).

The region between 1300 cm^{-1} and 1000 cm^{-1} in the spectra of representatives of (I) show many sharp peaks, some of which are due to the C-O and C-O-C stretching modes. Oxazolidine ring systems have three IR bands between 1200 cm^{-1} and 1100 cm^{-1} characteristic of the O-C-N group[150]; frequencies higher than these could indicate a contribution from O-C=N.
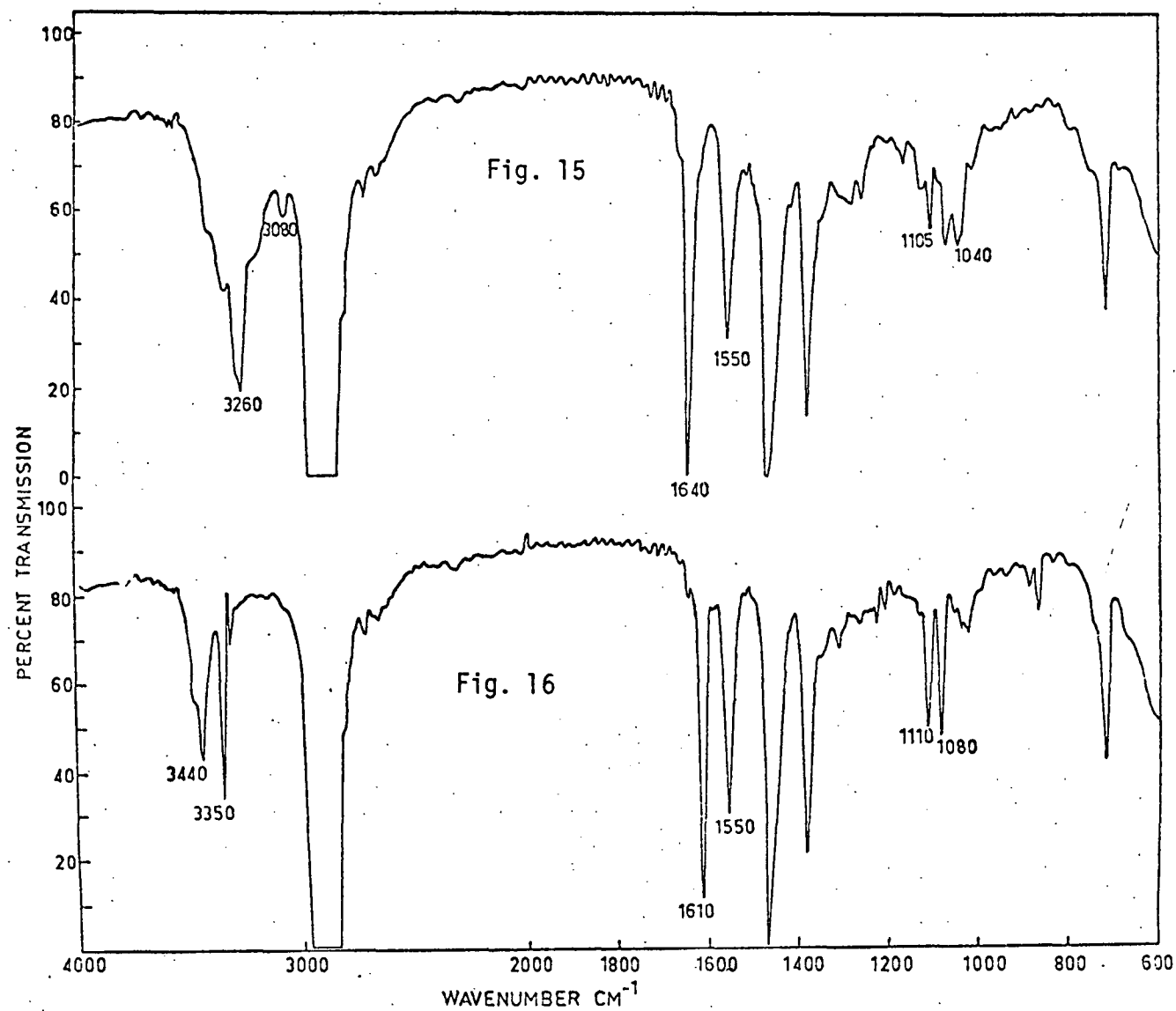
IR spectra of the diastereoisomeric ceramides, 24:0-DL<u>e</u>-d18:0 (Fig. 15) and 24:0-DL<u>t</u>-d18:0 (Fig. 16).
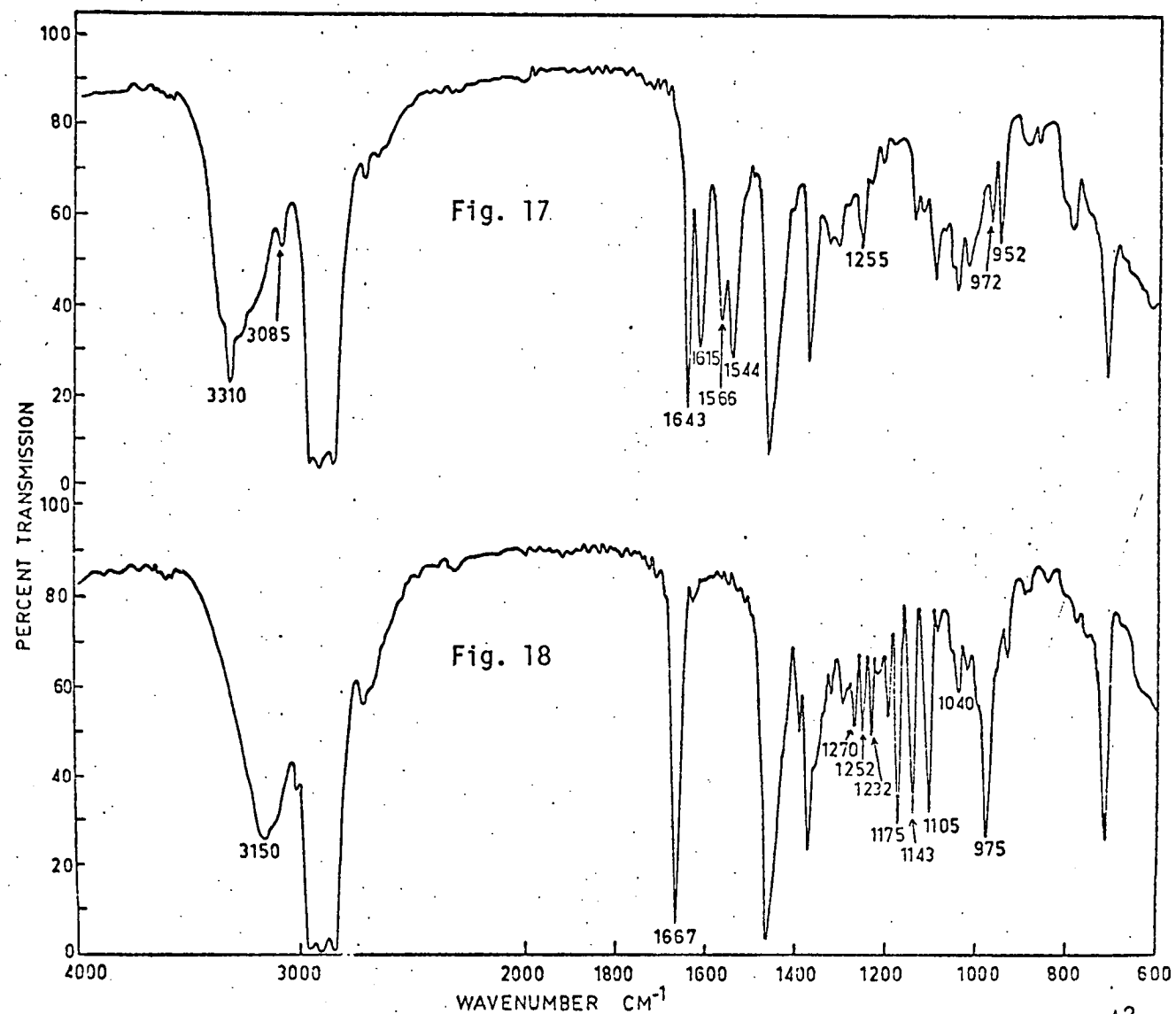
IR spectra of a ceramide, 14:0-De-d18:1 (Fig. 17), and the parent oxazoline, 2-Trid-4-(1h-Hexad$^{\Delta 2}$)-2-oxazoline (Fig. 18).
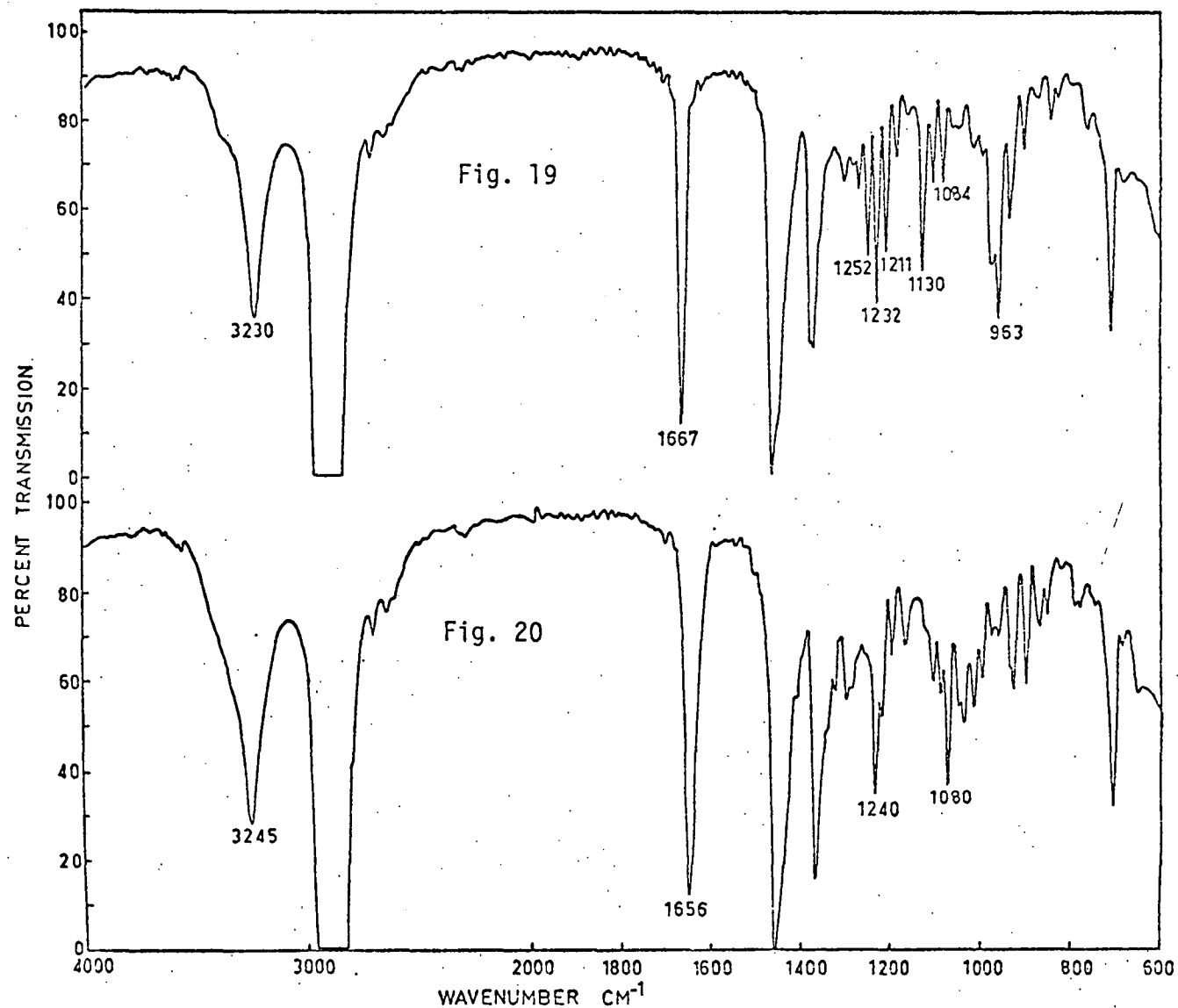
IR spectra of the isomeric oxazolines, 2-Trid-4-(1h-Hexad)-2-oxazoline (Fig. 19) and _trans_-2-Trid-4-hMe-5-Pentad-2-oxazoline (Fig. 20).

APPENDIX 1

SPHINGOLIPIDS FROM THE MILK OF FRIESIAN COWS

The methods for the isolation of the sphingomyelin and glycosylceramides from milk have been described in Chapter 1 (p.16); ceramides were isolated from the lipid fraction rich in these by preparative TLC. Sphingomyelin and monoglycosylceramides were converted to ceramides[58,92], which were separated by TLC into two classes, NFA and HFA derivatives; the former were separated again by TLC on plates impregnated with borate into two fractions, one containing saturated sphingoids and the other unsaturated sphingoids[71]. All the ceramide fractions, and the diglycosylceramides, were hydrolysed[98], and the methyl esters analysed by GLC by the method described in Chapter 1 (p.33). The mixture of normal and hydroxy methyl esters from the diglycosylceramides were separated by TLC prior to analysis.

The structures of the main glycosphingolipids of Friesian milk were shown to be glucosylceramides and lactosylceramides by comparison of their IR spectra with those of the glycosphingolipids from other milk samples[48,151], and by TLC on plates impregnated with borate[152]. The proportion of hydroxy fatty acids in these compounds was higher (6% ^{W}/w of the total) in whole Friesian milk than in buttermilk power (less than 1%)[153]; sphingomyelin contained less than 1% (^{W}/w) of hydroxy fatty acids. The fatty acid compositions of the sphingolipids are tabulated (Table 1), and the separation of the methyl esters derived from NFA sphingomyelins and HFA sphingo-myelins are illustrated (Figs. 1 and 2 respectively). The principal acids, in decreasing order of importance, of NFA sphingo-lipids are 23:0, 24:0, 22:0, and 16:0, and of HFA sphingolipids are 23:0,

h23:0, h24:0, h22:0 and h16:0. Such results have been observed by others for ceramides[103], sphingomyelin[154,155], glycosylceramides[93,153], and gangliosides[156], all obtained from either buttermilk powder or Holstein cow milk. The similarity in the composition of the sphingolipids suggests (a) that unsaturated sphingoids are derived from saturated sphingoids[157], and (b) that ceramides may be a common precursor in their biosynthesis, although the ceramides may have been partly derived from the sphingomyelins by enzymatic hydrolysis[40].

Table 1

Fatty acid composition (weight %) of the major sphingolipids from the milk of Friesian cows

Fatty acid	Ceramide	Sphingomyelin			Glucosyl ceramide			Lactosyl ceramide	
	NFA[a]	NFA[b]	NFA[c]	HFA[a]	NFA[b]	NFA[c]	HFA[a]	NFA[a]	HFA[a]
12:0	0.5	0.1	trace	0.1	-	-	-	1.1	trace
14:0	1.7	0.7	0.4	1.9	1.2	0.3	1.9	5.9	1.4
15:0	0.1	0.2	0.2	0.2	0.2	0.1	trace	0.4	0.1
16:0	16.8	14.0	15.8	8.3	9.8	10.8	9.7	16.6	13.5
16:1	0.1	0.2	0.1	-	0.3	0.1	-	0.5	-
17:0	0.5	0.3	0.3	-	0.3	0.2	0.5	0.4	0.8
18:0	3.6	2.9	2.2	3.9	2.8	1.3	3.8	10.4	4.2
i18:0	4.2	6.3	3.6	-	1.1	1.3	-	4.9	-
18:1	1.6	0.2	0.2	0.2	-	-	-	0.5	0.1
18:2	0.6	trace	0.1	-	-	-	-	-	-
19:0	0.3	0.8	0.4	0.6	0.4	0.2	trace	0.1	-
20:0	0.6	1.6	1.0	1.6	1.8	0.8	2.3	2.2	1.2
21:0	0.9	1.4	0.8	1.5	2.1	0.2	1.0	1.2	1.3
21:1	trace	0.2	trace	-	1.9	0.8	-	-	-
22:0	16.7	17.4	18.6	16.5	22.3	23.9	18.9	17.2	16.2
22:1	0.2	2.0	0.3	-	2.1	1.0	-	-	-

Table 1 (continued)

	a)	b)			b)			c)	
23:0	23.5	23.4	24.3	32.0	25.9	29.8	34.7	19.5	24.8
23:1	0.8	2.5	0.6	0.2	2.6	1.3	0.3	0.8	2.9
24:0	17.7	14.6	19.9	17.9	18.9	21.5	19.0	15.6	23.8
24:1	4.6	5.9	3.8	7.3	2.8	3.9	2.1	0.8	3.8
25:0	2.6	3.0	2.1	3.8	1.7	0.9	2.4	1.0	1.5
25:1	0.9	1.6	0.9	1.4	1.9	1.5	1.1	0.3	1.2
26:0	0.5	0.6	0.5	1.3	2.1	1.0	1.3	0.4	1.7
26:1	0.3	0.4	0.3	1.5	0.3	0.1	0.7	0.2	0.9
27:0	0.1	0.4	0.2	0.1	0.5	0.2	0.2	0.2	0.1
27:1	0.2	0.2	0.3	-	0.3	0.1	-	0.1	-
28:0	0.2	0.8	0.1	0.1	0.9	0.3	0.2	0.1	-
28:1	0.1	trace	0.2	-	trace	-	-	-	0.1
Total unsaturates	9.5	13.8	7.0	10.6	12.3	8.9	4.3	4.2	8.9

a) derivatives of total sphingoids.   b) derivatives of saturated sphingoids.   c) derivatives of unsaturated sphingoids.
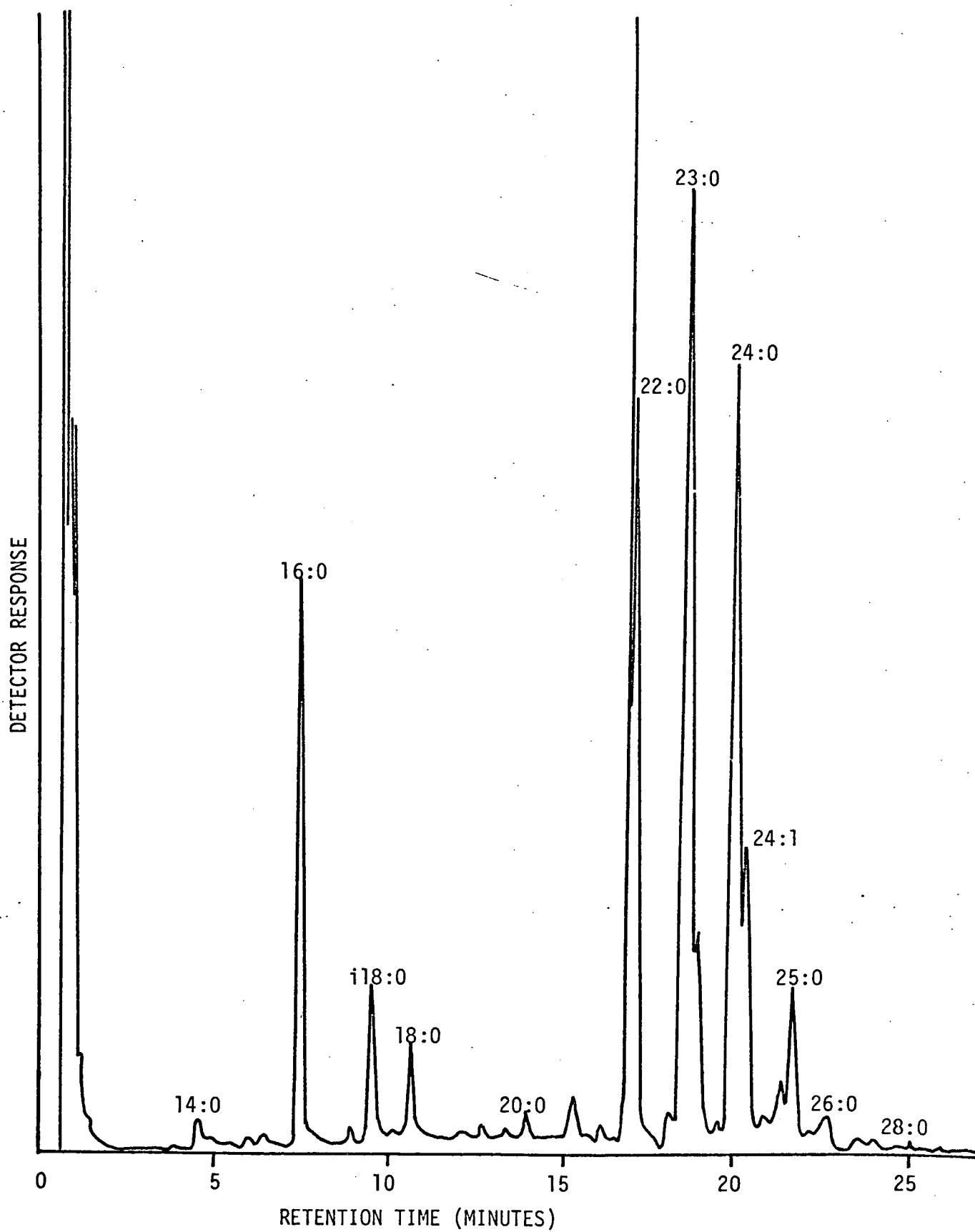
Fig. 1.   GLC of the normal methyl esters derived from bovine milk
sphingomyelin.   Operating conditions have been described in Ch. 1 (p.33).
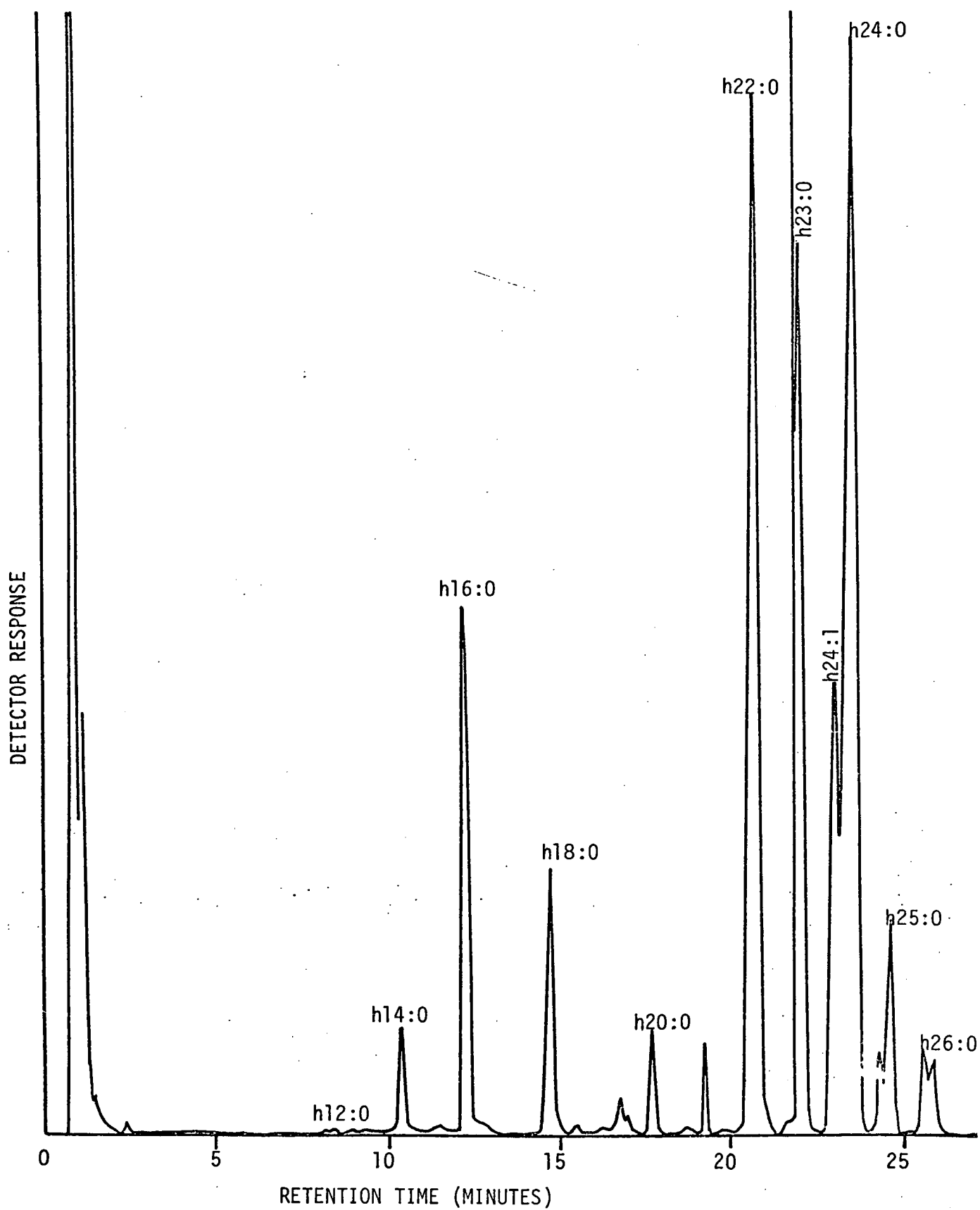
Fig. 2.   GLC of the 2-trimethylsiloxy methyl esters derived from bovine milk sphingomyelin.   Operating conditions have been described in Ch. 1 (p.33).

APPENDIX 2

SERUM LIPIDS IN WORKERS EXPOSED TO LEAD

Blood sera from 68 male adults from the Electrolytic Zinc Company (Hobart, Tas.) were analysed for cholesterol, free ceramide, and monoglyceride, by the spectrodensitometric method described in Chapter 1 (p. 29). The results are compared to those of normal adults not exposed to such industrial conditions (Table 1), and those of other normal surveys[79]. The mean values for free cholesterol and total cholesterol are similar to the normal values, but ceramides and monoglycerides are present in higher concentration in workers exposed to lead than in normals. These findings require further investigations which are outside the scope of this project; they appear in this thesis as an example of practical application of analytical methods treated in Chapter 1.

Table 1

Serum lipids of industrial employees exposed to heavy metals, in particular, lead

Number	NFA Ceramides (mg %)	Monoglycerides (mg %)	Free Cholesterol (mg %)	Total Cholesterol (mg %)	Total/Free Cholesterol
1	1.8	7	23	204	8.7
2	2.1	9	40	290	7.3
3	3.8	4	54	277	5.1
4	4.1	3	57	253	4.4
5	4.5	7	74	286	3.9
6	3.0	4	83	253	3.0
7	3.0	18	70	198	2.8
8	3.0	11	34	177	5.2
9	1.8	10	75	216	2.9
10	2.1	9	48	202	4.2
11	1.8	7	23	148	6.4
12	1.8	9	35	200	5.7
13	2.1	7	44	226	5.1
14	1	2	20	192	9.6
15	1.8	9	31	242	7.8
16	1	2	44	277	6.3
17	1	1	27	200	7.4

Table 1 (continued)

18	1	7	23	219	9.5
19	1	3	27	233	8.6
20	2.1	7	39	296	7.6
21	6.1	7	97	276	2.8
22	4.1	7	102	260	2.5
23	5.8	2	109	296	2.7
24	4.5	2	57	215	3.8
25	5.8	7	97	296	3.1
26	3.8	9	57	222	3.9
27	2.1	7	48	215	4.5
28	3.0	9	78	258	3.3
29	1.8	9	66	232	3.5
30	4.1	18	92	234	2.7
31	2.1	22	88	221	2.5
32	4.1	18	97	248	2.6
33	3.5	9	97	331	3.4
34	3.8	30	97	364	3.8
35	3.8	28	92	298	3.3
36	3.5	18	74	250	3.4
37	5.8	22	82	257	3.1
38	6.1	35	114	298	2.6
39	3.8	10	66	212	3.2

Table 1 (continued)

40	8.0	27	88	267	3.0
41	5.8	26	70	274	3.9
42	4.5	25	43	200	4.7
43	4.5	25	78	267	3.4
44	3.8	30	97	309	3.2
45	5.8	30	92	252	2.7
46	3.8	18	66	220	3.3
47	3.8	22	74	236	3.2
48	4.5	28	78	261	3.3
49	8.0	38	64	210	3.3
50	9.0	15	103	290	2.8
51	3.8	30	88	268	3.0
52	5.8	25	78	192	2.5
53	4.1	18	60	186	3.1
54	5.8	40	92	242	2.6
55	3.8	18	57	212	3.7
56	5.8	30	92	304	3.3
57	4.5	18	55	208	3.8
58	3.0	40	103	282	2.7
59	2.1	25	66	193	2.9
60	4.1	28	64	260	4.0
61	4.5	38	70	268	3.8

Table 1 (continued)

62	1	8	50	202	4.0
63	1.8	18	48	186	3.9
64	3.0	11	87	209	2.4
65	1	9	46	160	3.5
66	1.8	30	66	202	3.1
67	1	9	55	254	5.0
68	1	7	66	212	3.2
Mean ± ISD	3.6 ± 1.8	15.8 ± 10.5	67.3 ± 24.2	241 ± 42.2	3.6 ± 1.7
Control	2.7	7	63	217	3.4

APPENDIX 3

LIPOAMINO ACIDS

Since amino acids were found in lipid extracts in the course
of this work, compounds soluble in typical solvents for lipids, and
in which a single amino acid or peptide was covalently linked either
through the carboxyl or amino group to a neutral lipid moiety, were
studied under this heading. Proteolipids and lipopeptides[158], and
aminoacylphosphatides of the kind found in bacteria[159], and
phosphatidyl serine, will be excluded.

1.   Review

Investigations of the lipids of tissues and microorganisms
have not shown unequivocally the presence of lipoamino acids in all
cases.   The formation of lipid-amino acid complexes has, however,
been reported in cellular preparations, such as hen oviduct mince[160,161].
In general, the complex has been demonstrated after addition of
radioactive amino acid to the cell preparation, in a medium of
supporting metabolism, by the measurement of radioactivity in a crude
or purified fraction extracted with lipid solvents, and the subsequent
recovery of the unchanged amino acid after hydrolysis.   It was found
that the complexes could be separated into discrete fractions by
countercurrent distribution and column chromatography.   Furthermore,
the non-polar lipid fraction eluted from silicic acid with benzene
contained virtually no radioactivity; this argues against the
participation of neutral lipids in the complexes; it was suggested
that neutral lipids containing amino acids might well behave like
polar lipids[160].

The constituent lipids of whole human blood have been examined by chromatography on columns of silicic acid[162]. All lipid fractions, with the exception of the least polar fractions (cholesterol esters and triglycerides) liberated amino acids on hydrolysis. In a later paper by the same author, it was concluded that some lipid-amino acid complexes are artefacts formed in vitro from endogenous amino acids, but that others do exist in vivo[163].

The nature of the bonding between the amino acids and the lipids has been investigated[164]. To determine whether the carboxyl groups of the amino acids were covalently linked to the lipids by ester bonds, the reaction of various lipid-amino acid complexes with hydroxylamine were studied; the presence of both ester and possibly amide bonding was established. The presence of N-acylamino acids in skin has been suggested, but not proven[165].

2. Evidence for the existence of lipoamino acids

Proof for the existence of natural lipoamino acids could not be found by the use of TLC alone, although this technique did provide some evidence. For example, plates overloaded with natural lipids and then developed to separate the neutral ones gave some very faint positive ninhydrin reactions around cholesterol. The degree of reaction increased when the plates were sprayed with hydrochloric acid after development, heated for 30 minutes at 120°, and then sprayed with ninhydrin.

Stronger evidence was found by the use of GLC. The neutral lipid fractions (cholesterol esters, triglycerides, and cholesterol) and fatty acids of blood plasma (heart disease cases) were isolated by TLC, and treated with 6N hydrochloric acid overnight at 110°. The

lipid residues were removed by extraction with chloroform, and the aqueous phase filtered and evaporated to dryness. TLC was found inadequate for identifying the amino acids in the residue[166], and GLC, especially the method of Gehrke et. al.[87], was routinely used (p.37). The compositions of the lipid-bound amino acids were compared with those of the free amino acids of the plasma samples. There seemed to be no apparent order in the amino acid patterns of any of the lipid extracts, but the relative concentrations of some lipid-bound amino acids differed greatly from those of the free amino acids (Table 1). This is an indication that the amino acids are not present in lipids simply as artefacts, although the lipids may act as better solvents for the higher molecular weight species.

Table 1

Relative ratios of some free and lipid-bound amino acids in plasma.

Lipid class	Amino acid ratios		
	Lys/Try	Tyr/His	$\dfrac{Gly + Ala}{leu + ileu}$
free amino acids	1	1	1
cholesterol esters	0.66	1.45	10.98
cholesterol	0.26	0.88	12.72
triglycerides	0.51	0.42	1.56
fatty acids	-	0.23	3.64

3. The synthesis of lipoamino acids

(a) Cholestanol and cholesterol esters of amino acids

The preparation of cholestanol esters of neutral, non-hydroxy amino acids was carried out essentially by a method described by others, that of direct fusion of cholestanol with the amino acid in the presence of dry hydrogen chloride[167]. The product was dissolved in chloroform, and ammonia added to liberate the free ester from its salt. The ammonium chloride was washed out with water, the solvent removed, and the ester recrystallized from ethanol. Some of the compounds prepared are tabulated (Table 2).

Cholesterol esters of amino acids could not be synthesised by direct fusion, and the following method was used. Cholesterol (1 mmole), N-phthaloyl DL-alanyl chloride[168,169] (1 mmole) and triethylamine (1.45 mmole) were fused at 150° under nitrogen to give a high yield (70-80%) of cholesterol N-phthaloyl DL-alaninate (no product was formed when the reaction was carried out in pyridine). Recrystallization from ethanol afforded 300 mg of white crystals, m.p. 137-138°, and which gave a single spot (R_f 0.7) on a TLC plate developed with chloroform-methanol (98:2). The phthaloyl derivative (200 mg) was refluxed for one hour with hydrazine (0.3 mmole) in ethanol (30 ml), without appreciably affecting the ester bond. The phthaloyl hydrazide was removed, and the crude cholesterol DL-alaninate (120 mg) purified by TLC (developing solvent, chloroform-methanol (93:7)). The derivative was found difficult to obtain free of contaminating cholesterol and starting material, either by TLC, column chromatography, or crystallization. However, the hydrochloride salt (m.p. 232° (dec.)) could be isolated after treating an ethanolic solution of the free amine with 6 N hydrochloric acid. This compound was stable under normal laboratory conditions, but the

Table 2

Cholestanol and cholesterol esters of amino acids[a]

No.	Compound Name	M.p.°	Yield %	R_f value[b]	Formulae	C% Found (Calcd.)	H% Found (Calcd.)	N% Found (Calcd.)
1	cholestanol glycinate HCl[c]	245-6 (dec.)	48	0.45				
2	cholestanol glycinate[c]	178-180	47	0.45				
3	cholestanol alaninate HCl[c,d]	238 (dec.)	66	0.62	$C_{30}H_{54}O_2NCl$	71.6 (72.6)	11.0 (11.0)	2.8 (2.8)
4	cholestanol alaninate[c,e]	120	68	0.62	$C_{30}H_{53}O_2N$	78.2 (78.4)	11.6 (11.6)	3.0 (3.0)
5	cholesterol alaninate HCl[f]	138	30	0.62	$C_{30}H_{52}O_2NCl$	72.6 (73.6)	10.6 (10.8)	2.7 (2.8)
6	cholestanol prolinate HCl	224-7 (dec.)	65	0.49	$C_{31}H_{57}O_2NCl$	72.7 (73.6)	10.7 (10.8)	2.5 (2.7)
7	cholestanol prolinate	133-5	64	0.49	$C_{31}H_{56}O_2N$	79.2 (79.1)	11.4 (11.4)	2.9 (2.9)
8	cholestanol phenylalaninate HCl	226-8 (dec.)	42	0.74	$C_{35}H_{58}O_2NCl$	74.8 (75.5)	10.1 (10.2)	2.4 (2.5)
9	cholestanol glycylleucinate	98-100	32	0.35	$C_{35}H_{61}O_3N_2$	75.9 (75.4)	10.9 (11.0)	4.5 (5.0)

a) New compounds are underlined. b) Developing solvent, $CHCl_3$-CCl_4-CH_3OH-H_2O (50:50:10:0.5); R_f value for cholesterol or cholestanol, 0.68. c) Higher yields obtained here than reported[167]. d) Reported M.p. 258°(dec.)[167]. e) No analytical data given[167]. f) Analysis for Cl, Found 7.4; Calcd. 7.2.

Table 3

N-acylamino acids and N-acylpeptides, and derivatives related to glyceryl esters of amino acids[a]

No.	Compound Name	M.p.°	Yield %	R_f value[b]	Formulae	C% Found (Calcd.)	H% Found (Calcd.)	N% Found (Calcd.)
1	N-stearoyl DL-alanine[c]	109-110	84	0.76				
2	N-stearoyl DL-threonine	82-6	79	0.69				
3	N-stearoyl DL-glycylleucine[d]	103-5	65	0.78	$C_{26}H_{50}O_4N_2$	67.2 (67.7)	11.1 (11.1)	6.3 (6.2)
4	1-(N-phthaloyl DL-alanyl)-2,3-propenoate	38.5	89		$C_{14}H_{13}O_4N$	64.8 (64.9)	5.1 ((5.1)	5.6 (5.4)
5	1-(N-phthaloyl DL-alanyl)-2,3-epoxypropane	61-3	73		$C_{14}H_{13}O_5N$	61.4 (61.1)	5.1 (4.8)	5.3 (5.1)
6	1-(N-phthaloyl DL-alanyl)-propane-2,3-diol[e]	-	45		$C_{14}H_{15}O_6N$	56.4 (57.3)	5.6 (5.2)	4.7 (4.8)

a) All compounds, with the exception of No. 1, are new.   b) Developing solvent, $CHCl_3$-CH_3OH-H_2O (65:25:4);   the N-acyl derivatives move with cerebrosides.   c) This compound has the same properties as the active one reported, i.e. N-stearoyl L-alanine[170].   d) The IR spectrum shows absorption at 3335 cm^{-1} and 3310 cm^{-1} (ν N-H), 1735 cm^{-1} (ν C=O), 1650 cm^{-1} and 1618 cm^{-1} (amide I), 1544 cm^{-1} and 1513 cm^{-1} (amide II), and 1263 cm^{-1} and 1245 cm^{-1} (amide III). e) This compound could not be induced to crystallize.

free amine deteriorated over several weeks in cold chloroform.

(b) <u>N-acylamino acids</u>

N-acylamino acids and N-acylpeptides were synthesised by the method of Suyama et al.[170], and purified by recrystallization from chloroform or hexane-chloroform mixtures. The IR spectra of the compounds tested have a band at 3310 cm^{-1} (ν N-H), which would probably be characteristic of all N-acylamino acids except the basic ones. The positions of the bands due to the ester and amide groups vary. Some of the compounds tested have been analysed (Table 3, Nos. 1 to 3).

(c) <u>Glyceryl esters of amino acids</u>

The preparation of glyceryl esters of neutral, non-hydroxy amino acids was not possible by direct fusion methods. The reaction between α-monostearin and alanine in the presence of dry hydrogen chloride produced mainly distearin and glycerol. A reaction scheme similar to that for the production of cholesterol esters of amino acids was attempted, but the final step of removing the phthaloyl group with hydrazine, either hot or cold, resulted in the destruction of the ester bond. However, some new compounds were prepared (Table 3, Nos. 4 to 6) which may be useful for further work of this nature.

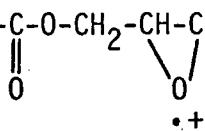(ı) <u>1-(N-phthaloyl DL-alanyl)-2,3-propenoate</u>

N-phthaloyl DL-alanyl chloride (10.6 g), allyl alcohol (35 ml, a 0.25 M excess), and pyridine (30 ml) were refluxed under nitrogen for 1 hour, cooled, and the solution poured into an ice-cold saturated sodium bicarbonate solution (100 ml) with vigorous stirring. A light yellow oil settled out, which was extracted into ether (2 x 100 ml),

washed with water and 10% sodium chloride solution, and dried over anhydrous sodium sulphate. The ether was removed under reduced pressure, and the residual oil dissolved in a minimum volume of hot petroleum ether (60-80). On cooling, a colourless oil reprecipitated, which was crystallized by shaking vigorously in an ice-salt bath. The white product obtained was chromatographically pure, and showed the desired spectral characteristics.

(ii) 1-(N-phthaloyl DL-alanyl)-propane-2,3-diol

Hydroxylation of the double bond in the ester (i) was carried out in hot hydrogen peroxide-acetic acid, a method which has been used successfully both with and without a catalyst for the hydroxylation of allyl alcohol[171]. The allyl ester (4 mmole) was dissolved in glacial acetic acid (4 ml), hydrogen peroxide (8 mmole) added and the solution heated on a water bath at 80° for two days. Practically all of the allyl ester had reacted after this time, as judged by TLC, and two products of R_f values 0.30 and 0.60 (developing solvent, $CHCl_3-CH_3OH$ (92:8)) were separated. Both compounds were colourless viscous liquids which could not be induced to crystallize. The product corresponding to R_f 0.30 was shown spectrally to be 1-(N-phthaloyl DL-alanyl)-glycerol (yield 0.53 g) and it gave a positive periodate-Schiff's reaction on the plate indicating the 1,2-diol group[76]. The product corresponding to R_f 0.60 (yield 0.4 g) gave a negative periodate-Schiff's test, and was determined to be an acetate (formed probably during the reaction via the ion, $R-\overset{\overset{\parallel}{O}}{C}-O-CH_2-\overset{O}{\overset{\diagdown\diagup}{CH}}-\overset{+}{CH_2}$) by analysis of its spectra, e.g. a methyl singlet at 7.9 τ in the NMR spectrum and a sharp OH stretch in the IR spectrum (3470 cm^{-1}).

A second method for hydroxylation was carried out on a small scale using osmium tetroxide[172]; in this case the acetate was not formed, and TLC indicated the major reaction product the same as above.

(iii) 1-(N-phthaloyl DL-alanyl)-2,3-epoxypropane

Epoxidation of the double bond in the ester (i) was carried out with m-chloroperbenzoic acid[173]. The allyl ester (0.01 mole) and metachloroperbenzoic acid (2.5 g of 83%) in dichloromethane (30 ml) were stirred overnight at room temperature (21°). Titration of an aliquot with standard sodium thiosulphate solution indicated 35% reaction. A little catalyst was now added (18 μl of trifluoroacetic acid), the temperature raised to 27°, and the reaction allowed to continue for three days. After this time 70-80% reaction had occurred. The excess peracid was destroyed with sodium sulphite (0.76 g) and the epoxide freed from m-chlorobenzoic acid by washing with 5% sodium bicarbonate solution, followed by 10% sodium chloride solution and finally water. The solvent was evaporated, and the residual oil crystallized from petroleum ether (60-80)-ethyl ether (9:1) at -10°. It showed one TLC spot only, gave a positive periodate-Schiff's test, and its spectra indicated the epoxide.

(d) N-aminoacyl sphingoids

N-phthaloyl DL-alanyl chloride (1 mmole), DL(e+t)-d18:0 (1 mmole) and triethylamine (160 μl) in dimethylformamide (2 ml) were allowed to stand overnight at room temperature. Ether (200 ml) was added, the mixture washed with water, and the solvent removed under reduced pressure. N-phthaloyl DL-alanyl-DL(e+t)-d18:0, m.p. 98-104°, was isolated by preparative TLC (developing solvent, chloroform-methanol

(9:1)) in 80% yield. The phthaloyl group was removed by treatment with hydrazine (0.8 mmole) in alcohol (3 ml) at 40° for two days. The diastereoisomeric mixtures, L-alanyl-DL($\underline{e+t}$)-d18:0, and D-alanyl-DL($\underline{e+t}$)-d18:0, were isolated from the reaction mixture by TLC (developing solvent, chloroform-methanol (3:1) ; yield, 28% of each); the R$_f$ values were 0.35 and 0.27 respectively. Their structures were confirmed by TLC comparison with the products derived from either L- or D-alanine, and by IR spectroscopy (3280 cm^{-1}, 3080 cm^{-1}, 1642 cm^{-1}, 1255 cm^{-1}, 1075 cm^{-1}, and vibrations due to C-H for both compounds; the L- and D-isomers were distinguished by adsorption at 1525 cm^{-1} and 1550 cm^{-1} respectively). On TLC plates impregnated with borate, compounds containing $\underline{e}$-d18:0 were separated from those containing $\underline{t}$-d18:0.

The compound, DL-alanyl-D$\underline{e}$-d18:1, prepared by the above method on a smaller scale, gave similar chromatographic and spectral properties as described for the saturated analogues.

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